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Volume 34

Fusion of Biological Membranes and Related Problems

Edited by

Herwig Hilderson
and Stephen Fuller

Fusion of Biological Membranes and Related Problems

Subcellular Biochemistry
Volume 34

SUBCELLULAR BIOCHEMISTRY

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eBook ISBN: 0-306-46824-7
Print ISBN: 0-306-46313-X

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New York, Boston, Dordrecht, London, Moscow

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Contributors

Tina Ballegaard Institute of Biochemistry, Odense University, 5230 Odense M, Denmark

Pad N. Black Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, New York 12208

Ineke Braakman Department of Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Prodyot K. Chatterjee Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

Philippe Collas Institute of Medical Biochemistry University of Oslo, PO Box 1112 Blindern, 0317 Oslo, Norway

Hilde De Busser RUCA-Laboratory for Human Biochemistry, Department of Biochemistry, Physiology and Genetics, University of Antwerp, B-2020 Antwerp, Belgium

Werner P. De Potter UIA Laboratory for Neuropharmacology Department of Medicine, University of Antwerp, B-2610 Wilrijk-Antwerp, Belgium

Concetta DiRusso Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, New York 12208

Ben Distel Department of Biochemistry Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Robert W. Doms Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

- Ype Elgersma** University of California at Los Angeles, Department of Neurobiology, Los Angeles, California 90095
- Joakim Faergeman** Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, New York 12208
- Dieter Gallwitz** Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany
- Yves Gaudin** Laboratoire de Génétique des Virus, CNRS, 91198 Gif sur Yvette Cedex, France
- Don L. Gibbons** Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461
- Martin Götte** Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany
- Cordula Harter** Biochemie-Zentrum Heidelberg, Ruprecht-Karls-Universität, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany
- Catherine L. Jackson** Division of Biochemistry and Molecular Genetics, Department of Cellular and Molecular Biology, SBGM, Bat 142, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France
- Margaret Kielian** Department of Cell Biology, Albert Einstein College of Medicine, 1300 Moms Park Ave., Bronx, New York 10461
- Jens Knudsen** Institute of Biochemistry, Odense University, 5230 Odense M, Denmark
- Helmut Kubista** Department of Physiology, University College London, Gower Street, London WC1E 6BT, United Kingdom
- Albert Lagrou** RUCA-Laboratory for Human Biochemistry, Department of Biochemistry, Physiology and Genetics, University of Antwerp, B-2020 Antwerp, Belgium
- Thomas Lazar** Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany
- Michal Linial** Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, 91904 Israel
- Yanping E. Lu** Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

Carrie M. McManus Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Stephen Moss Department of Physiology, University College London, Gower Street, London WC1E 6BT, United Kingdom

Peter Partoens UIA-Laboratory for Neuropharmacology, Department of Medicine, University of Antwerp, B-2610 Wilrijk-Antwerp, Belgium

Dominic Poccia Department of Biology, Amherst College, Amherst, Massachusetts 01002

Constanze Reinhard Biochemie-Zentrum Heidelberg, Ruprecht-Karls-Universität, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany

Sandra Sacre Department of Physiology, University College London, Gower Street, London WC1E 6BT, United Kingdom

David Sanders Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Dietrich Scheglmann Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany

Dirk Slembrouck UIA Laboratory for Neuropharmacology, Department of Medicine, University of Antwerp, B-2610 Wilrijk-Antwerp, Belgium

Henk F. Tabak Department of Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Guido Van Dessel UIA-Laboratory for Pathological Biochemistry, Department of Medicine, University of Antwerp, B-2020 Antwerp, Belgium

Peter F. T. Vaughan Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, United Kingdom

Jin-San Yoo Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany

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Preface

Membrane fusion and targetting processes are tightly regulated and coordinated. Dozens of proteins, originating from both the cytoplasm and membranes are involved. The discovery of homologous proteins from yeast to neurons validates a unified view.

Although much is known about the interfering proteins, the events occurring when two lipid bilayers actually fuse are less clear. One cannot exclude that lipid bilayers behave like soap-bubbles fusing when meeting each other. In this respect interfering proteins should be considered as preventing undesirable and unnecessary fusion and eventually directing the biological membrane fusion process (when, where, how and overcoming the activation energy).

In this volume of the series *Subcellular Biochemistry* some aspects of fusion of biological membranes as well as related problems are presented. Although not complete, one will find a lot of recent information including on virus-induced membrane fusion. The contributors of the chapters are all among the researchers who performed many of the pioneering studies in the field.

A first glance to the subject is found in *Chapter 1*, written by Cardula Harter and Constanze Reinhard. They give an overview of the route of a protein along the secretory pathway and summarize the progress that was made within the last decades. They discuss the current models for the formation and fusion of vesicular carriers with a major focus on the mechanism underlying budding of a COPI-coated vesicle.

In *Chapter 2* Michal Linial describes how neurotoxins (α -Latroxin and Clostridial toxins) can be used as tools in dissecting the exocytic machinery. The mode of action of α -Latroxin (inducing massive and uncontrolled vesicular release of neurotransmitters and neuropeptides) is still not

known. The effect of Clostridial toxins (blocking neurotransmitter release causing neuromuscular paralysis) on exocytosis can be explained by their activities on the SNAREs. According to the author, structural information on SNARE and on their associated proteins should be evaluated in view of the biophysics and kinetic properties of fusion. Therapeutic applications are described.

The role of annexins (a distinct family of Ca^{2+} -binding proteins) in vesicular traffic and membrane fusion events in exocytosis and endocytosis is discussed by Helmut Kubista, Sandra Sacre and Stephen Moss in *Chapter 3*. The authors state that many of the properties involved in NSF, SNAPs, SNAREs-dependent vesicle transport and membrane fusion machinery are shared by annexins. It is possible that annexins provide a specialized exocytotic pathway that works in parallel to other mechanisms, such as NSF-dependent exocytosis. Alternatively, some data suggest that annexins may function together with the NSF, SNAPs and SNARE-machinery. Some annexins are membrane fusogens, others may be involved in transport to and aggregation of vesicles at fusion sites, in regulation of the cytoskeleton at these sites, in organization of fusion membranes, in regulation of other annexins and in Ca^{2+} signaling.

In *Chapter 4* Martin Götte *et al.* show how the entire information of baker's yeast *Saccharomyces cerevisiae* genome is now known and freely accessible. All membrane fusion in eukaryotic cells is governed by a class of monomeric GTP-binding proteins, called Rab in mammals and Ypt in yeast. After a brief introduction to the Ras-superfamily the reader is familiarized with the structural features and posttranslational modifications common to all Ypt proteins. The functional cycling and the various states of Ypt proteins as well as the proteins known to interact with the Ypt GTPases are discussed. Current models of the function of every single member of the Ypt proteins in the vesicular transport are reviewed (Ypt1p, Yp31p, Ypt32p, Sec4p for exocytic and Ypt51p, Ypt52p, Ypt53p, Ypt7p for vacuolar and endocytic trafficking). The authors conclude that three Ypt GTPases (Ypt1p, Ypt3p, Sec4p) suffice in regulating trafficking from the ER to the plasma membrane, while three proteins (Ypt51p, Ypt6p, Ypt7p) mediate vesicular transport on the endocytic pathway.

In *Chapter 5* Nils Faergeman *et al.* highlight the numerous effects of long-chain fatty acyl-CoA esters (through partitioning into membranes, acyl-CoA-dependent remodeling in vesicle trafficking or reversible acylation of proteins) as cofactors for the fission and fusion of biomembranes. The significance of these effects is discussed in relation to the physiological concentration of long-chain fatty acyl-CoA esters and their binding proteins.

Brefeldin A (BFA) is known to prevent association of ARF to Golgi membranes and to inhibit secretion. In *Chapter 6* Catherine Jackson

discusses the early work describing the effects of BFA at both morphological and molecular levels. Next she introduces the family of Sec7 domain ARF exchange factors, and discusses the studies showing that they are major targets of BFA both *in vitro* and *in vivo*. The implications of the recently elucidated mechanism of action of BFA on Sec7 domain ARF exchange factors in transport through the ER-Golgi system are reviewed. Finally the author places ARF and its regulators into a model for ER-through-Golgi transport that takes into consideration the diverse *in vivo* effects of BFA.

The membrane fusion events during nuclear envelope assembly is reviewed by Philippe Collas and Dominic Poccia in *Chapter 7*. Several issues are addressed, including the multistep assembly of the nuclear envelope (from nuclear vesicles), the biochemical requirements for fusion of nuclear vesicles, evidence for the involvement of small GTPases in nuclear vesicle fusion, analogies between nuclear vesicle fusion and fusion events in intracellular membrane trafficking, the controversial role of Ca^{2+} in nuclear envelope assembly and evidence that some nuclear vesicles may harbor specific fusogenic elements.

A genetic approach to study the various processes involved in the maintenance, biosynthesis and proliferation of peroxisomes, creating a wealth of new ideas and information, experimental tools and mutants are discussed in *Chapter 8* by Ben Distel *et al.*

Neurons, chromaffin cells and membrane fusion are the subject of *Chapter 9*, the result of the collaboration between three different research groups (Hilde De Busser *et al.*; Peter Partoens *et al.*; Peter Vaughan). The authors limit themselves to a description of the membrane composition of the large dense-cored vesicles (LDVs)/secretory granules from which it can be concluded that the crucial actors governing small synaptic vesicles (SSVs) exocytosis are also responsible for LDV exocytosis. Subsequently they emphasize the role of the cytoskeleton in the exocytosis of LDVs and the role of GTP-binding proteins. Finally the related isoprenylating and carboxymethylating mechanisms are highlighted.

Yves Gaudin describes in *Chapter 10* how rhabdovirus-induced membrane fusion is an exception to the rule that the fusogenic protein in most cases of virus-plasma membrane fusion is synthesized in a metastable conformation, using the energy released during the fusogenic structural transition to achieve the energetically expensive membrane-fusion reaction. For rhabdoviruses the low pH-induced structural transition is absolutely reversible. A plausible role for the fusion inactive state is to avoid undesirable fusion in the acidic Golgi vesicles during the transport of fusogenic glycoprotein. The reversibility would be necessary for the protein being incorporated in a native functional conformation in neosynthesized virions.

The specific roles for lipids in virus fusion and exit is discussed by Margaret Kielian in *Chapter 11* using alphaviruses as an example. An efficient alphavirus exit requires the virus spike and nucleocapsid, the specific interaction of the E2 tail with nucleocapsid, the expression of 6K and correct lateral spike proteins interactions. In order to understand the roles played by cholesterol (entry, exit) and sphingolipids (hemifusion, complete fusion) in the alphavirus lifecycle (by interacting with viral proteins promoting fusogenic conformational changes) the current molecular understanding of alphavirus membrane fusion is discussed in detail. At the end of the article the author considers the available data suggesting a specific role of lipids in the entry and exit of other viruses and pathogens.

Chapter 12 is devoted to the fusion mediated by the HIV-1 envelope protein. In this chapter Carrie McManus and Robert Doms discuss the current understanding of the viral (Env, Gp120, Gp41) and cellular components (CD4, major and alternative HIV-1 coreceptors) necessary for the viral entry process. The identification of the HIV coreceptors coupled with detailed structural information on CD4, gp120 and gp41 has suggested several new anti-viral approaches. In addition to the coreceptors, Env itself can be targeted. Triggered or partially triggered Env proteins may elicit antibodies to highly conserved but poorly immunogenic domains that are competent to neutralize diverse virus isolates.

In the last chapter (*Chapter 13*) David Sanders shows how sulfhydryl groups are involved in fusion mechanisms. Cysteine is a special residue because it is sensitive to the oxidation/reduction potential of its environment. The author states that proteins promoting intracellular membrane fusion reside in the reducing environment of the cytoplasm, whereas those participating in membrane fusion between enveloped viruses and cells or between two or more cells are exposed to an oxidizing milieu. Following discussion of protein thiols in cellular membrane fusion and in viral-glycoprotein-mediated membrane fusion and virus entry, the author reconsiders the alphavirus entry, incorporating both acidified endosomes and thiol-disulfide exchange.

Finally, we would like to thank Paul Van Dyck for assisting in preparation of this book.

We wish all of you much enthusiasm and pleasure in reading the book.

Antwerp, Belgium
Heidelberg, Germany

Herwig Hilderson
Steve Fuller

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Chapter 1

The Secretory Pathway From History to the State of the Art

Cordula Harter and Constanze Reinhard

1. SUMMARY

Maintenance of the structural and functional organization of a eucaryotic cell requires the correct targeting of proteins and lipids to their destinations. This is achieved by the delivery of newly synthesized material along the secretory pathway on one hand and by the retrieval of membranes on the other hand. Various models have been suggested over the years to explain traffic flow within the secretory pathway. The only two models that are under discussion to date are the “vesicular model” and the “cisternal maturation model”. A wealth of information from various experimental approaches, strongly supports the vesicular model as the general mode of intracellular transport.

Three major types of protein-coated transport vesicles are characterized in molecular detail, and have been attributed to various steps of the secretory pathway: COPII-coated vesicles allow exit from the endoplasmic reticulum (ER), COPI-coated vesicles carry proteins within the early

CORDULA HARTER and CONSTANZE REINHARD Biochemie-Zentrum Heidelberg, Ruprecht-Karls-Universität, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany. *Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems*, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

secretory pathway, i.e. between ER and Golgi apparatus, and clathrin-coated vesicles mediate transport from the *trans*-Golgi network (TGN).

In this review we will give an overview of the route of a protein along the secretory pathway and summarize the progress that was made within the last decades in the characterization of distinct intracellular transport steps. We will discuss the current models for the formation and fusion of vesicular carriers with a major focus on the mechanism underlying budding of a COPI-coated vesicle.

2. DEFINITION OF THE SECRETORY PATHWAY

2.1. Discovery

Discovery of the secretory pathway is closely connected to the history of the Golgi apparatus. After establishing a new staining method (black reaction, “reazione nera”) (Golgi, 1898) to visualize cellular structures at the light microscope level, Camillo Golgi noticed a basket like network surrounding the nucleus of Purkinje cells. He published the first description of this structure in 1898 (Golgi, 1898), calling it “apparato reticolare interno”. The name was shortened to “Golgi apparatus” or “Golgi complex”. In the following fifty years, it was hotly debated whether this structure corresponds to an authentic organelle or a preparation artifact. Reason for that was Golgi’s difficult heavy-metal staining method that was hard to reproduce reliably and stained many other structures. As long as cell structures have been investigated only by conventional light microscopy, the “Golgi controversy” could not be clarified. It was resolved with the development of the electron microscope. The first studies at the ultrastructural level by Dalton and Felix (1954) and Sjöstrand and Hanzon (1954) described the Golgi complex as a stack of paired, smooth-surfaced lamellae with a characteristic curvature surrounded by vacuoles of different size. This structure was seen in the same region of a cell where the Golgi complex was detected by Golgi staining with light microscopy.

That the Golgi complex may be related to secretion was actually reported as early as 1902 by Fuchs (1902) who accurately described a structure in exocrine cells closely associated with secretory granules. However, Fuchs did not relate his observation to Golgi’s initial description that was already published.

Although it had long been recognized that the Golgi complex was highly developed in secretory cells, it was not until the 1960s that its role in secretion became clear. Once again, it was the introduction of new techniques, in this case metabolic labeling, cell fractionation and EM-

autoradiography which meant a great progress to science. Using these methods, Palade and coworkers (Palade, 1975) obtained complementary biochemical and morphological data delineating vectorial transport of secretory proteins through the cell, involvement of the Golgi complex and of vesicular intermediates.

2.2. Stations of the Secretory Pathway

2.2.1. Endoplasmic Reticulum

The entry station for all proteins of the secretory pathway (this includes secretory proteins, plasma membrane proteins and proteins destined for various compartments of the endomembrane system) is the endoplasmic reticulum (ER). The ER is the largest endomembrane system within eucaryotic cells and performs a wide variety of functions. Classically, the ER is known to be composed of three morphologically distinct subcompartments: nuclear envelope, rough ER and smooth ER. More recently, it has been suggested that the ER may be further divided into specialized subdomains that are distinct in terms of their protein constituents, morphological appearance and functions (Hobman *et al.*, 1998; Montero *et al.*, 1997; Nishikawa *et al.*, 1994; Sitia and Meldolesi, 1992).

The journey of a typical secretory or membrane protein begins with the targeting to the ER membrane of the nascent polypeptide chain by a hydrophobic signal sequence. The signal sequence interacts with the signal recognition particle complex, and the growing polypeptide chain is translocated across the ER membrane to the ER lumen or inserted into the ER membrane (reviewed in: Kalies and Hartmann, 1998; Wilkinson *et al.*, 1997). On the luminal site of the ER various chaperones associate with the nascent polypeptide chain in order to control and support correct folding (reviewed in: Bukau and Horwich, 1998; Hartl, 1996). In addition, the newly synthesized protein undergoes co- and post-translational processing, i.e. glycosylation, disulfide bond formation, and oligomerization in some case. Once the protein is properly “matured”, it passes the quality control of the ER and exits this compartment via coated vesicles.

2.2.2. ER-Golgi Intermediate Compartment

Protein transport from the ER to the Golgi complex involves the ER-Golgi intermediate compartment (ERGIC), also described as 15°C compartment (Saraste and Kuismanen, 1984) and vesicular tubular clusters (VTCs) (Balch *et al.*, 1994). The ERGIC has originally been defined morphologically. Lowering the temperature to 15°C led to a concentration of

proteins in the Golgi area en route to the plasma membrane (Schweizer *et al.*, 1990; Saraste and Kuismanen, 1984). Biochemically, this structure is characterized by the small GTPase Rab2 (Tisdale and Balch, 1996; Chavrier *et al.*, 1990) and a glyco-membrane protein called ERGIC-53 in human (Schindler *et al.*, 1993; Schweizer *et al.*, 1988) and p58 in rat (Saraste *et al.*, 1987). Several experiments have shown that ERGIC-53/p58 cycle within the early secretory pathway and localize at steady state to the ERGIC. However, the nature of this structure is unclear. At least three models are discussed: (i) the ERGIC is physically connected with the ER and consists of tubular extensions emanating from the ER membrane (Krijnse-Locker *et al.*, 1994; Sitia and Meldolesi, 1992); (ii) the ERGIC is formed by fusion of ER-derived transport vesicles, and thus represents a transient structure that may eventually mature to the *cis*-Golgi network (Bannykh and Balch, 1997; Lippincott-Schwartz, 1993; Saraste and Svensson, 1991), and (iii) the ERGIC is a distinct organelle separated from the ER and the Golgi (Klumperman *et al.*, 1998; Hauri and Schweizer, 1992; Schweizer *et al.*, 1990). Although the nature of the ERGIC is discussed controversially, it is generally accepted that the ERGIC plays a role as a sorting station of retrograde and anterograde membrane flow (Scales *et al.*, 1997; Tisdale *et al.*, 1997; Aridor *et al.*, 1995; Pepperkok *et al.*, 1993).

2.2.3. The Golgi Apparatus

En route through the secretory pathway, proteins enter the Golgi at its *cis*-side (*cis*-Golgi network, CGN). They continue their passage through the several subcompartments of the Golgi (CGN, *cis*-, *medial*-, *trans*-Golgi and *trans*-Golgi network (TGN)), where they are subjected to various kinds of post-translational processing, e.g. remodeling of the *N*- and *O*-linked oligosaccharide side chains (Roth, 1997). Processing of carbohydrates occurs in an ordered sequence of enzyme-catalyzed reactions according to the directed transport through the Golgi complex.

Main element of the *cis*-compartment is a network of membranous tubules, usually seen in close contact and parallel to the *medial*-Golgi (Rambourg *et al.*, 1974). Typical marker enzymes for the *cis*-Golgi are the GlcNAc-Phosphotransferase catalyzing the first step of mannose 6-phosphate signal attachment (Kornfeld and Kornfeld, 1985), and the Peptide-Gal/NAc-transferase which initiates *O*-glycosylation (Roth *et al.*, 1994; Schweizer *et al.*, 1994).

The *medial*-Golgi consists of a number of more or less flattened cisternae or saccules close to each other to form stacks typical for the Golgi. Each cell type seems to contain a characteristic number of such saccules, usually between 3–11 (Rambourg and Clermont, 1997). GlcNAc-

transferase I involved in remodeling of *N*-glycosylation is one of the marker molecules for the *medial*-Golgi (Dunphy *et al.*, 1985).

The *trans*-compartment is composed of 3–6 superposed sacculo-tubular elements which, in contrast to elements of the medial Golgi, do not remain strictly parallel to each other. One of the last processing steps before proteins exit from the Golgi complex are sialylation and tyrosine sulfation catalyzed by the sialyltransferase (Roth *et al.*, 1985) and by the Tyrosylprotein-sulfotransferase (Huttner, 1988), respectively.

The exit site of the Golgi, the *trans*-Golgi network (TGN), is a highly variable structure depending on the cell type. It has been suggested that the TGN itself is composed of functionally distinct subdomains that might be involved in sorting of cargo proteins into different types of transport vesicles destined for endosomes, lysosomes, secretory granules and the plasma membrane (Matter and Mellman, 1994; Burgess and Kelly, 1987; Griffiths and Simons, 1986).

3. TRANSPORT THROUGH THE SECRETORY PATHWAY

The various transport steps between the stations of the secretory pathway require the packaging of cargo in some kind of containers that bud from a donor compartment and fuse with an acceptor compartment. A number of models have been proposed over the years to explain traffic flow along the secretory pathway. The only two that are still under discussion are the “vesicular model” and the “cisternal maturation model”.

The vesicular model—already suggested by Palade (1975)—predicts that small vesicles carry cargo between distinct compartments. In order to allow sequential processing of carbohydrates and sorting of cargo to the correct destination, vesicle flow must be directional, i.e. a vesicle budding from the ER specifically fuses with the *cis*-Golgi, a vesicle from the *cis*-Golgi fuses with cisternae from the *medial*-Golgi, etc. (reviewed in: Rothman and Wieland, 1996; Schekman and Orci, 1996).

A completely different view on the mode of intracellular trafficking is based on the transport of molecules that may be too large in order to be packaged into small vesicles, such as algal scales (Becker *et al.*, 1995) and procollagen (Bonfanti *et al.*, 1998). In this case, it has been suggested that transport occurs through progressive maturation of the Golgi cisternae (Mironov *et al.*, 1997). According to this model, the cargo molecules would be transported without leaving the lumen of a Golgi cisternae, i.e. without being packaged into a separate transport vesicle. Exit from the ER is suggested to be mediated by tubules elongating directly from the ER surface (Bonfanti *et al.*, 1998).

However, a wealth of information from biochemical, morphological and genetic approaches strongly supports the vesicular model as the general mode of intracellular transport. It may well be that the cisternal maturation model applies to some kind of cargo in specialized cells.

In the following sections, we will focus on the major types of transport vesicles: (i) COPII-coated vesicles for export from the ER; (ii) COPI-coated vesicles for transport into and through the Golgi in anterograde direction as well as transport between the Golgi and the ER in retrograde direction, and (iii) clathrin-coated vesicles for transport between the TGN and the plasma membrane (Figure 1).

3.1. Export from the ER

Export from the ER is the first step in the vectorial movement of cargo through the secretory pathway in eucaryotic cells. Originally, distinct exit sites in the ER for proteins and lipids have been defined morphologically in specialized secretory cells (Orci *et al.*, 1994; Palade, 1975). Specialized export complexes of the ER have been suggested to exist in all eucaryotic cells and to consist of small vesicles and tubular structures that may fuse with one another thus giving rise to the ERGIC. Based on quantitative immunocytochemistry and an evaluation of cargo, it is believed that vesicular tubular structures represent the sites where sorting and concentration of cargo destined for ER export occurs (Bannykh *et al.*, 1996; Balch *et al.*, 1994; Mizuno and Singer, 1993).

3.1.1. COPII-Coated Vesicles

The generation of transport intermediates from the ER is driven by the recruitment of a set of soluble proteins from the cytoplasm to the ER membrane that form a coat structure, termed COPII (Barlowe *et al.*, 1994, reviewed in: Barlowe, 1998). Components of the COPII coat have initially been characterized by use of a collection of Sec (Sec for secretion) mutants of *S. cerevisiae* which are defective at distinct steps in intracellular transport (Kaiser and Schekman, 1990). This led to the identification of three soluble protein components that make up the coat of ER-derived transport vesicles in yeast: Sar1p, Sec23p complex and Sec13p complex (Table I). Sar1p is a GTPase that shares primary structure identity with the Ras family of GTPases (Barlowe *et al.*, 1993; Nakano and Muramatsu, 1989). The Sec23p complex migrates at 400 kDa and is composed of two proteins: Sec 23p, an 85 kDa protein, and a tightly associated protein of 105 kDa, called Sec24p (Hicke *et al.*, 1992). The Sec 13p complex has a molecular mass of 700 kDa and contains the 34 kDa Sec13p subunit (Pryer *et al.*, 1993) and the

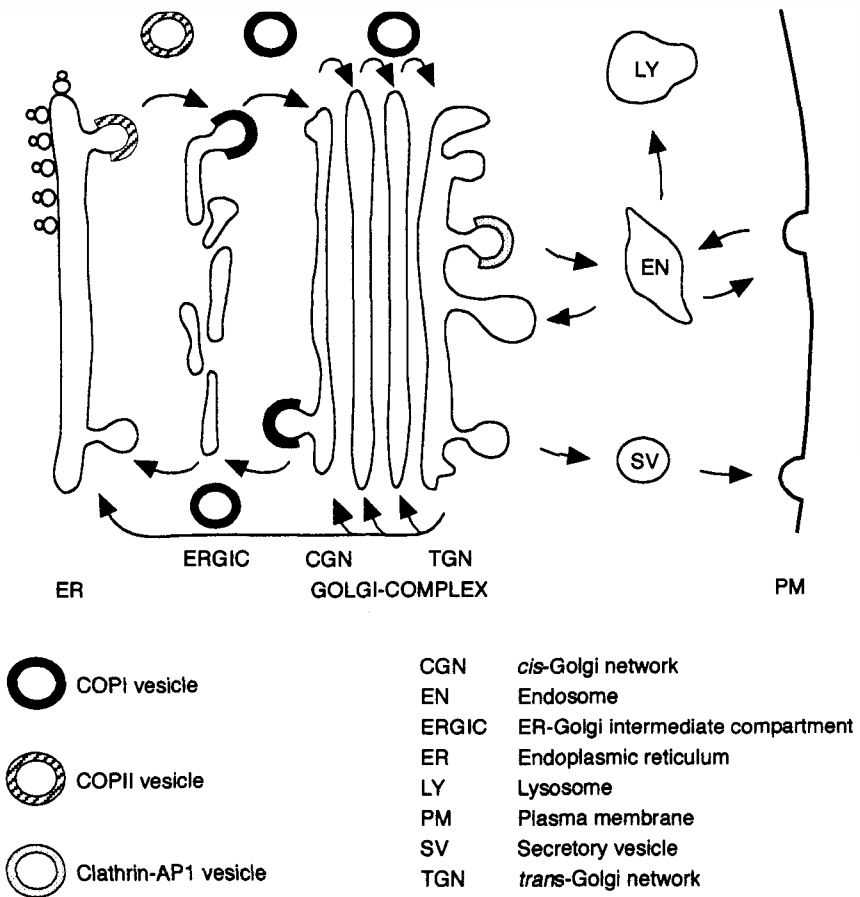


FIGURE 1. Model of vesicular transport along the secretory pathway in a mammalian cell. Export from the ER to the ERGIC occurs in COPII-coated vesicles. Transport from the ERGIC to the *cis*-side of the Golgi and through the Golgi apparatus is mediated by COPI-coated vesicles. COPI vesicles are also involved in retrograde vesicle traffic from the Golgi to the ER. Sorting at the exit site of the Golgi—the *trans*-Golgi network—to organelles of the endocytic pathway and to the plasma membrane can be mediated by clathrin-AP1-coated vesicles. Sorting at the TGN may also involve other types of transport vesicles. For details and references see text.

150 kDa subunit Sec31p (Salama *et al.*, 1997; Salama *et al.*, 1993). Both Sec13p and Sec31p contain repeated WD 40 motifs, conserved stretches of approximately 40 amino acids terminating at the residues Trp, Asp, that are generally thought to mediate protein-protein interactions (Neer *et al.*, 1994).

In vitro reconstitution experiments using washed microsomes have allowed to dissect the formation of COPII vesicles (Salama *et al.*, 1993). The initial step involves recruitment of Sar1p-GTP to the ER membrane through a guanine nucleotide exchange reaction catalyzed by the integral membrane protein Sec12p (Barlowe and Schekman, 1993). Next, Sec23p complex is recruited, a prerequisite for binding of Sec13p complex. Functionality of *in vitro* generated COPII vesicles was demonstrated by their capability to fuse with Golgi membranes. Fusion requires prior dissociation of the coat. Uncoating is achieved by hydrolysis of GTP bound to Sar1p, a reaction catalyzed by Sec23p, part of the coat complex (Yoshihisa *et al.*, 1993). This leads to the dissociation of Sar1p from the vesicle and a destabilization of the coat. In the presence of non-hydrolyzable analogues of GTP, COPII proteins are retained on the vesicle, and thus targeting and fusion is prevented (Barlowe *et al.*, 1994).

Mammalian homologues of Sar1p (Kuge *et al.*, 1994), Sec13p (Shaywitz *et al.*, 1995) and Sec23p (Paccaud *et al.*, 1996; Orci *et al.*, 1991) have been identified on the molecular level and, as expected, have been demonstrated to play a role in COPII coat formation at the ER membrane of mammalian cells, similar to yeast (Aridor *et al.*, 1998).

In vitro reconstitution experiments have clearly shown that the three cytosolic components Sar1p, Sec13p and Sec23p are all that is needed to generate a functional COPII-coated vesicle (Barlowe *et al.*, 1994). *In vivo*, two other proteins, Sec16p (Espenshade *et al.*, 1995) and Sed4p (Gimeno *et al.*, 1995), have been implicated in vesicle budding from the ER. SEC16 encodes a 240 kD protein that is peripherally associated with the ER membrane and also detected in ER-derived vesicles. Sec16p has been shown to interact with both Sec23p and Sec24p and has been proposed to act as a scaffold for membrane association of the cytosolic coat proteins (Gimeno *et al.*, 1996). It may also be involved in protein sorting since temperature sensitive Sec16p mutants show defects in selective packaging of specific cargo molecules (Campbell and Schekman, 1997). Sed4p is highly homologous to Sec12p (45% identity when the cytoplasmic domains are compared) but is not essential under standard growth conditions and does not catalyze the nucleotide exchange rate of Sar1p as was observed for Sec12p. Sed4p binds Sec16p and it is thought that together with Sar1p a multimeric protein complex is formed that is involved in a certain aspect of COPII vesicle budding (Gimeno *et al.*, 1995).

3.2. Transport into and through the Golgi

Transport of material from the ER to the *trans*-side of the Golgi involves passage through the intermediate compartment and the various subcompartments of the Golgi. In fact, vesicular membrane flow was first reconstituted in a cell free system by using isolated Golgi membranes (Balch *et al.*, 1984b). Coated structures were found to bud preferentially from the rims of Golgi cisternae. It is now well established that intra-Golgi transport is mediated by the recruitment to the Golgi membrane of soluble proteins from the cytoplasm, termed COPI. More recently, it was shown that COPI is also involved in transport from the ERGIC to the *cis*-side of the Golgi (reviewed in: Lowe and Kreis, 1998).

Several experimental data support their function in anterograde transport within the early secretory pathway: (i) Biosynthetic cargo such as albumin and VSV-G protein (Serafini *et al.*, 1991b), or reporter molecules carrying a signal for anterograde transport (Nickel *et al.*, 1998) have been detected in isolated COPI vesicles. Furthermore, a subpopulation of COPI-coated vesicles has been characterized morphologically and shown to contain insulin (Orci *et al.*, 1997). (ii) Quantitative immuno-EM has shown that the majority of β -COP can be detected at the *cis*-side of the Golgi (Oprins *et al.*, 1993). This is consistent with extensive co-localization of COPI with biosynthetic cargo (VSV-G) on the ERGIC (Griffiths *et al.*, 1995). After a short release from a 15°C block, VSV-G is trapped in COPI vesicles. (iii) Transport of VSV-G to the Golgi can be blocked by antibodies against a subunit of the COPI coat (Pepperkok *et al.*, 1993; Peter *et al.*, 1993); (iv) COPI can be recruited to pre-Golgi intermediates where it initiates segregation of VSV-G protein (Aridor *et al.*, 1995), and (v) several mutants of *S. cerevisiae* that are defective in secretion show defects in one of the COPI subunits (Duden *et al.*, 1994; Hosobuchi *et al.*, 1992).

3.2.1. COPI-Coated Vesicles

In vitro generation of COPI-coated vesicles from isolated Golgi membranes has allowed their biochemical characterization (Ostermann *et al.*, 1993; Serafini *et al.*, 1991b). Components that make up the coat of COPI vesicles are the cytosolic proteins ADP-ribosylation factor 1 (ARF1) (Serafini *et al.*, 1991a) and coatamer, a hetero-heptameric protein complex (Waters *et al.*, 1991) (Table 1).

ARF1, like Sar1p of the COPII coat, belongs to the family of Ras GTPases. The coatamer complex migrates at 600 kDa and consists of seven different subunits, called α - (Gerich *et al.*, 1995), β - (Serafini *et al.*, 1991b);

Table 1
Coat Proteins of Vesicles of the Secretory Pathway

Protein Complex	Subunits		Size	Features / Interactions
	Mammals	Yeast	Mammals	
COPII				
Sec13 complex	hSec13p	Sec13p	~34 kDa	WD-40 repeats
	hSec31p	Sec31p	~150 kDa	WD-40 repeats
Sec23 complex	hSec23p	Sec23p	~85 kDa	GAP for Sar1p Sec16p
	hSec24p	Sec24p	~105 kDa	Bos1p, Bet1p
Sar1	hSar1p	Sar1p	~21 kDa	Ras GTPase / Bos1p
COPI				
Coatomer	α -COP	Ret1p	~140 kDa	WD-40 repeats / β' -COP, ϵ -COP
	β -COP	Sec26p	~107 kDa	δ -COP, ARF1
	β' -COP	Sec27p	~102 kDa	WWD-40 repeats / α -COP
	γ -COP	Sec21p	~97 kDa	ζ -COP, ARF1, KKXX, p24 family
	δ -COP	Ret2p	~57 kDa	β -COP
	ϵ -COP	Sec28p	~35 kDa	α -COP
	ζ -COP	Ret3p	~20 kDa	γ -COP
ARF 1	ARF 1	yARF	~20 kDa	-Ras GTPase / β -COP, γ -COP,
Clathrin-Adaptor 1				
AP1 complex	γ -Adaptin	Ap14p	~100 kDa	σ 1-Adaptin
	β 1-Adaptin	Ap12p	~100 kDa	μ 1-Adaptin, Clathrin hc, di-Leu-based motifs
Clathrin	μ 1-Adaptin	Apm1p	~50 kDa	β 1-Adaptin, Tyr-based motifs
	σ 1-Adaptin	Aps1p	~19 kDa	γ -Adaptin
	3 hc*	CHC1	~190 kDa	β 1-Adaptin, hsc70, β -arrestin
	3 Ic ^{Δ}	CLC1	~30–40 kDa	
	ARF 1	yARF	~20 kDa	Ras-GTPase
Clathrin-Adaptor 3				
AP3 complex	δ -Adaptin	Ap15p	~160 kDa	
	β 3-Adaptin	Ap16p	~120 kDa	Clathrin, di-Leu-based motifs
	μ 3-Adaptin	Amp3p	~50 kDa	Tyr-based motifs
	σ 3-Adaptin	Ams3p	~25 kDa	
Clathrin	3 hc*	CHC1	~190 kDa	β 3-Adaptin, hsc70, β -arrestin
	3 Ic ^{Δ}	CLC1	~30–40 kDa	

* heavy chain

^Δ light chain

Duden *et al.*, 1991), β' - (Harrison-Lavoie *et al.*, 1993; Stenbeck *et al.*, 1993), γ - (Harter *et al.*, 1996), δ - (Faulstich *et al.*, 1996), E- (Hara-Kuge *et al.*, 1994), ζ -COP (Kuge *et al.*, 1993) (COP for coat protein). Each subunit seems to be present in a stoichiometric ratio of one by one in both the cytosolic form of coatamer as well as in its membrane-associated form. Only ζ -COP is found in the cytoplasm in a monomeric state (Kuge *et al.*, 1993). The function of monomeric ζ -COP is unknown. Interestingly, several COPs display homologies both in size and primary sequence to adaptins of the clathrin-coated vesicles: β -COP with β -/ β' -adaptins (Duden *et al.*, 1991; Serafini *et al.*, 1991b), δ -COP with μ -adaptins (Faulstich *et al.*, 1996) and ζ -COP with σ -adaptins (Kuge *et al.*, 1993). However, no functional homologies between clathrin-adaptor complexes and coatamer have been identified so far. Another homology exists between α - and β' -COP: their N-terminal domains contain, respectively, four and five WD-40 repeated motifs, similar to Sec13p and Sec31p of COPII.

Genetic analysis has identified yeast homologues to all of the coatamer subunits (Table I). Homologues of β -, β' -, γ - and ε -COP in *S. cerevisiae* are the products of the SEC26 (Duden *et al.*, 1994), SEC27 (Duden *et al.*, 1994), SEC21 (Hosobuchi *et al.*, 1992) and SEC28 (Duden *et al.*, 1998) genes, respectively. These genes have been isolated in a screen for mutants that are defective in the secretory pathway. It has been shown that these mutants exhibit a temperature-sensitive defect in transport between the ER and the Golgi apparatus. In a genetic screen designed to identify mutants defective in the retrieval of ER-resident proteins from the cell surface, the yeast homologues of mammalian α -, δ - and ζ -COP were identified and designated Ret1p (Letourneur *et al.*, 1994), Ret2p (Cosson *et al.*, 1996) and Ret3p (Cosson *et al.*, 1996) (Ret for retrieval), respectively. Based on the high degree of conservation between mammalian COPs and their yeast homologues, and the finding that yeast mutants show transport defects in both the anterograde and retrograde direction it is likely that coatamer, and, thus, COPI vesicles, are involved in bidirectional transport pathways in both yeast and mammalian cells (see 3.4).

Similar to COPII, the formation of COPI-coated vesicles is initiated by recruitment of a GTPase (ARF1) from the cytoplasm to the membrane (Palmer *et al.*, 1993; Donaldson *et al.*, 1992a). Golgi membrane binding and activation of ARF1 is triggered by the exchange of GDP for GTP, a reaction that requires catalysis by a GTP-exchange factor (Donaldson *et al.*, 1992b; Helms and Rothman, 1992). It is suggested that binding of GTP to ARF1 results in a change of the conformation of the myristylated N-terminus which allows membrane association (Kahn *et al.*, 1992). Several nucleotide exchange factors for ARF1 have been characterized to date, all of which are soluble proteins found in the cytoplasm (reviewed in: Chardin

and McCormick, 1999; Moss and Vaughan, 1998). Some of these proteins require the presence of phosphoinositides for their catalytic activity (Meacci *et al.*, 1997; Chardin *et al.*, 1996). This led to the suggestion that activation of ARF1 at the correct membrane is regulated by specific nucleotide exchange factors that are recruited from the cytoplasm to the membrane. This is in contrast to the situation in the COPII system where nucleotide exchange on Sar1p is catalyzed by the integral ER membrane protein Sec12p (Barlowe and Schekman, 1993). Another important difference between COPII and COPI is the GTP hydrolysis reaction needed for uncoating prior to fusion. Whereas for COPII disassembly GTP hydrolysis on Sar1p is catalyzed by the coat protein Sec23p (Yoshihisa *et al.*, 1993), acceleration of GTP hydrolysis on ARF1 requires the activity of ARF-specific GAPs that are recruited from the cytoplasm to the Golgi membrane (Poon *et al.*, 1999; Brown *et al.*, 1998; Premont *et al.*, 1998; Cukierman *et al.*, 1995). Interestingly, structural and functional analysis of an ARF1-ARFGAP complex has revealed a role for coatamer as effector for efficient GTP hydrolysis (Goldberg, 1999). In GTPase assays using a soluble form of ARF1 (lacking the myristylated N-terminus), the addition of coatamer was found to accelerate the rate of GTP hydrolysis by a factor of 1000 in an ARFGAP-dependent manner. This led to the suggestion that a tripartite complex of ARF1, ARFGAP and coatamer exists on the surface of a membrane (Goldberg, 1999). A role of coatamer as effector for efficient GTP hydrolysis fits to the finding that in the ARF1-ARFGAP complex the effector site is open, and therefore accessible to coatamer (Goldberg, 1999). Resolution of the structure of this ternary complex will help to learn how ARF and coatamer work together in COPI vesicle formation.

3.3. Sorting at the *trans*-Golgi Network

Along their route through the Golgi, secretory and membrane proteins destined for various post-Golgi pathways are intermixed. Thus, proteins of distinct routes, i.e. endosomal and the secretory route, are likely to be sorted into various types of transport vesicles at the TGN (Keller and Simons, 1997; Traub and Kornfeld, 1997; Griffiths and Simons, 1986). Among the best-characterized types of TGN-derived vesicles are clathrin-coated vesicles, the formation of which seems to resemble that of COP-coated vesicles (reviewed in: Schmid, 1997). In addition, several types of non-clathrin-coated vesicles have been identified. Their exact functions remain to be elucidated (Gleeson *et al.*, 1996; Narula and Stow, 1995; Jones *et al.*, 1993).

3.3.1. Clathrin-Coated Vesicles

Already in 1967, it was suggested that hydrolytic enzymes are delivered to lysosomes from the Golgi in a certain population of coated vesicles (Friend and Farquhar, 1967). These Golgi-associated vesicles had a similar morphology to the bristle-coated vesicular structures observed at the plasma membrane of oocytes upon uptake of yolk protein (Roth and Porter, 1964). Biochemical characterization of both plasma membrane- and Golgi-derived vesicles was made possible by their purification from a variety of tissues (Pearse, 1975), and extraction of their coat proteins (Keen, 1987; Pearse and Robinson, 1984). It is now known that their major coat constituents are clathrin and various types of adaptor complexes (reviewed in: Hirst and Robinson, 1998; Schmid, 1997).

At least two types of TGN-associated adaptors have been identified to date and are believed to exert specific functions in transport to endosomes and lysosomes, respectively: one adaptor complex is called AP1 (Ahle *et al.*, 1988; Robinson, 1987) and the other AP3 (Dell'Angelica *et al.*, 1997) (Table I). A novel type of adaptor complex (AP4) which is believed to participate in sorting at the TGN has been described recently (Dell'Angelica *et al.*, 1999). However, it remains to be clarified whether this complex also interacts with clathrin. In all known clathrin-adaptor-coated vesicles, clathrin seems to provide the structural basis for coated vesicle formation (Vigers *et al.*, 1986; Unanue *et al.*, 1981). Clathrin assembles in triskelions that consist of three heavy chains of approximately 190kDa and three light chains of 3–40 kDa (Ungewickell and Branton, 1981). Adaptors are heterotetrameric protein complexes composed of two subunits with a molecular mass of about 100kDa (γ - and β 1- adaptin in AP1, δ - and β 3-adaptin in AP3), one medium chain of approx. 50kDa (μ 1- and μ 3-adaptin), and a small chain of approx. 20 kDa (σ 1- and σ 3-adaptin). The β -subunits of both AP1 and AP3 are thought to interact with clathrin via their N-terminal domains (Gallusser and Kirchhausen, 1993).

Information on the recruitment of AP1 was obtained by studies with isolated Golgi membranes (Stamnes and Rothman, 1993; Robinson and Kreis, 1992). These studies have shown that AP1 binding is dependent on the presence of ARF1-GTP, similar to what has been observed for the recruitment of coatomer to Golgi membranes. It is believed that binding of ARF1-GTP to Golgi membranes creates high-affinity binding sites for AP1, thus promoting coat assembly (Zhu *et al.*, 1998). But in contrast to COPI vesicle formation (see 4.1), ARF1-GTP is suggested to act in a process before budding and not to represent a stoichiometric coat component of clathrin-AP1-coated vesicles (Zhu *et al.*, 1998).

Other differences between COP-coated and clathrin-coated vesicles concern vesicle release and the uncoating event. Release of a clathrin-coated vesicle has been suggested to involve the activity of the GTPase dynamin which, in contrast to other GTPases, like ARF and Sar1p, is believed to act as a mechanochemical device responsible for membrane fission at the base of the coated bud (Jones *et al.*, 1998; reviewed in: Schmid *et al.*, 1998; for another view on dynamin action see: Kirchhausen, 1999; Sever *et al.*, 1999). Disassembly of the clathrin coat is believed to depend on the chaperone hsc70 (Schlossman *et al.*, 1984) and auxilin (Holstein *et al.*, 1996).

3.4. Recycling Pathways

Continuous vesicle flow in anterograde direction would ultimately lead to a decrease of the surface of the donor membrane and a depletion of its membrane proteins. At the same time the target membrane would grow and become enriched in these membrane proteins. Thus, proteins and lipids must be recycled from each station of the secretory pathway in order to maintain the structural and functional integrity of the endomembrane system.

Based on the current model of the secretory pathway (Figure 1), two major recycling pathways can be defined: (i) recycling within the early secretory pathway, i.e. from the various cisternae of the Golgi back to the ER (Jackson *et al.*, 1993; Lippincott-Schwartz *et al.*, 1990), and (ii) recycling within the late secretory pathway, i. e. from the plasma membrane to the TGN (reviewed in: Mellman, 1996; Gruenberg and Maxfield, 1995).

Retrograde transport between the Golgi and the ER is mediated by COPI-coated vesicles (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994), whereas internalization from the plasma membrane involves clathrin-coated vesicles (Boll *et al.*, 1996; reviewed in: Schmid, 1997). Since the topic of this review is the secretory pathway, we will focus on retrieval from the Golgi. For a detailed overview on recycling from the plasma membrane, the interested reader may refer to excellent previous reviews (Schmid, 1997; Mellman, 1996).

One function of retrograde transport is the retrieval of proteins that normally localize to the ER but escaped from their resident organelle. For many ER proteins it is known that sorting into retrograde vesicles is mediated by specific targeting motifs. The best-characterized ER-targeting signals are the KDEL-sequence of luminal proteins (Pelham, 1990; Munro and Pelham, 1987) and the KKXX- / KXKXX-motif of type I membrane proteins (Jackson *et al.*, 1990; Nilsson *et al.*, 1989), both localized at their C-termini.

The finding that coatomer is able to bind the C-terminal KKXX ER-retrieval motif was the first indication that COPI-coated vesicles may play a role in retrograde transport (Cosson and Letourneur, 1994). This was further supported by the following experimental data: (i) functional studies in yeast have revealed that several COPI mutants are deficient in retrograde transport of proteins carrying a KKXX signal from the Golgi to the ER (Cosson *et al.*, 1996; Letourneur *et al.*, 1994); (ii) reporter molecules bearing a KKXX retrieval signal are packaged in COPI-coated transport vesicles generated *in vitro* in the presence of GTP (Nickel *et al.*, 1998), and (iii) the KDEL receptor was detected as a major component of COPI-coated vesicles in mammalian cells pointing to a role of these vesicles in retrieval of luminal ER residents from the Golgi (Soennichsen *et al.*, 1996). Consistent with this, a population of COPI-coated vesicles has been identified by immuno-EM that selectively carries the KDEL-receptor as a marker for retrograde cargo but is devoid of anterograde cargo (Orci *et al.*, 1997).

Taken together, the experimental data discussed in 3.2 and above strongly support a role of COPI-coated vesicles in both anterograde and retrograde vesicular transport. It will now be important to identify the factors that determine the direction of a COPI vesicle.

3.5. Membrane Proteins in Vesicle Formation and Cargo Selection

Formation of coated vesicles is likely to require interaction of cytoplasmic domains of certain integral membrane proteins that may serve as coat receptors with the soluble coat components. Likewise, interaction of cytoplasmic domains of membrane cargo with coat components may result in their selective packaging in a certain type of transport vesicle. Sorting of soluble cargo requires involvement of transmembrane receptors which may couple sorting in the lumen of an organelle to coat assembly at the cytoplasmic face. The expected properties of a transmembrane cargo receptor include one or more transmembrane domains, a luminal domain able to interact with cargo species, and a cytoplasmically exposed domain that interacts with coat subunits. Further, such proteins would be expected to cycle between the donor and the acceptor organelle.

3.5.1. Membrane Proteins of COP-Coated Vesicles

Sorting of membrane cargo into a COPII pre-budding complex was described for several proteins in yeast (Kuehn *et al.*, 1998) and mammals (Rowe *et al.*, 1996; Aridor *et al.*, 1995), and is believed to be mediated via an interaction with the Sec23p complex. However, no direct binding of

these components has been reported. An acidic motif—D/EXD/E (two acidic amino acids separated by a variable residue)—in the cytosolic tail of several transmembrane cargo proteins has been suggested to serve as a sorting signal for ER export (Nishimura and Balch, 1997).

Sorting into COPI vesicles was suggested for membrane proteins carrying a KKXX-retrieval signal (see 3.4). This is based on the finding that fusion proteins or immobilized peptides containing such a signal at their C-termini are able to bind to coatomer or coatomer subcomplexes *in vitro* (Fiedler *et al.*, 1996; Harter *et al.*, 1996; Cosson and Letourneur, 1994; Letourneur *et al.*, 1994).

For sorting of soluble cargo, two types of membrane proteins are known to date that fulfill the criteria as cargokoat receptors. One is the KDEL receptor, a multi-spanning membrane protein that mainly localizes to the Golgi, and recognizes a carboxy-terminal tetrapeptide (KDEL). The KDEL-peptide has been shown to serve as a retrieval signal of soluble proteins that have escaped from the ER. Interaction of the KDEL-receptor with KDEL-bearing proteins retrieves them back to the ER (Lewis and Pelham, 1992) (see 3.4).

Another type of vesicular transmembrane proteins is referred to as the p24 family some members of which have been found in COPII- (Schimmoeller *et al.*, 1995) and COPI-coated vesicles (Sohn *et al.*, 1996; Stamnes *et al.*, 1995). These type I membrane proteins share a common structural organization: a large luminal domain with the propensity to form coiled-coils, and a short cytoplasmic domain that contains two conserved motifs: a di-phenylalanine motif and in most cases a di-basic motif at their C-termini.

In yeast, two members of the p24 family, Emp24p (Schimmoeller *et al.*, 1995) and Erv25p (Belden and Barlowe, 1996), have been localized to ER-derived COPII-coated vesicles. These proteins form a complex that is required for efficient ER to Golgi transport of a subset of secretory proteins (Belden and Barlowe, 1996). It is believed that Emp24p/Erv25p interact via their luminal coiled-coils with some cargo proteins, such as pre-pro- α -factor, thereby serving as cargo receptors for sorting into ER-derived COPII vesicles (Kuehn *et al.*, 1998; Schimmoeller *et al.*, 1995). However, no direct interaction of these domains with cargo molecules has been demonstrated so far.

Homologues of Emp24p and Erv25p have also been identified in mammals and are designated p24 (Stamnes *et al.*, 1995) and p23 (Rojo *et al.*, 1997; Sohn *et al.*, 1996), respectively. p24 and p23 were the first transmembrane proteins to be identified in COPI-coated vesicles (Sohn *et al.*, 1996; Stamnes *et al.*, 1995). Both proteins are especially abundant in Golgi membranes and are concentrated into Golgi-derived COPI-coated vesicles

where they are present in approximately stoichiometric amounts relative to coatamer and ARF. Thus, p24 and p23 are likely to be necessary for COPI-dependent budding. This is supported by two other findings: (i) the cytoplasmic domains of p23 and p24 are able to bind to coatamer (Fiedler *et al.*, 1996; Sohn *et al.*, 1996), and (ii) both proteins cycle within the early secretory pathway (Nickel *et al.*, 1997; Fiedler *et al.*, 1996). More recently, it was shown that p23 and p24, in addition to their COPI binding, are also able to bind to COPII *in vitro* (Dominguez *et al.*, 1998), and to form hetero-oligomeric complexes with various family members (p25, p26, and p27) (Gommel *et al.*, 1999; Dominguez *et al.*, 1998). COPII binding was suggested to involve their di-phenylalanine motif, whereas interaction with COPI depends on both their di-phenylalanine and di-basic motifs (Dominguez *et al.*, 1998; Sohn *et al.*, 1996). Based on these observations it is tempting to hypothesize that their state of oligomerization may regulate the interaction of p24 complexes with a certain type of coat and thus define whether p24 proteins are active in COPII or COPI vesicles (Gommel *et al.*, 1999).

Taken together, the above data have made p24 proteins strong candidates to act as coat receptors, whereas their putative role as cargo receptors is less clear. Although in yeast several lines of evidence support a role in selective sorting (Kuehn *et al.*, 1998; Schimmoeller *et al.*, 1995), a direct interaction with cargo molecules remains to be demonstrated.

3.5.2. Membrane Proteins of Clathrin-Coated Vesicles

Coupling of coat assembly and cargo selection is best understood for clathrin-coated vesicles. In this section we will briefly summarize the basic knowledge of membrane sorting at the TGN. For a detailed discussion of this topic, the reader may refer to several previously published, excellent reviews (Le Borgne and Hoflack, 1998; Kirchhausen *et al.*, 1997; Schmid, 1997). The best known example for a cargo-coat receptor is the mannose 6-phosphate receptor of TGN-derived clathrin-coated vesicles (Le Borgne and Hoflack, 1997). On the luminal side, the receptor recognizes the mannose 6-phosphate tag of lysosomal hydrolases. On the cytoplasmic face, the receptor tail interacts with AP1 thus initiating coat assembly (Glickman *et al.*, 1989). Sorting of membrane proteins into TGN-derived clathrin-coated vesicles has been shown to depend on a tyrosine-based sorting motif (YXXF, where F is a bulky hydrophobic amino acid) similar to the signal for internalization via AP2 at the plasma membrane. Both sorting at the TGN and endocytosis have been shown to be mediated via the μ -chain of the adaptor complexes (Ohno *et al.*, 1995).

4. MECHANISM OF VESICLE FORMATION. INSIGHTS FROM THE COPI SYSTEM

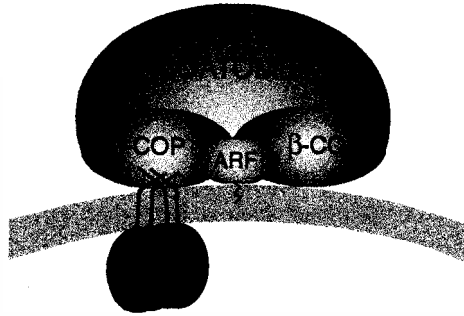
As outlined in the previous sections, assembly of the COPI coat on the surface of a donor membrane is initiated by the recruitment of activated ARF1. Subsequent binding of coatomer involves an interaction with trans-membrane proteins serving as coat receptors.

4.1. Bivalent Interaction of Coatomer

An issue that is discussed controversially, is whether ARF1 represents a structural component of COPI-coated vesicles or whether it acts catalytically in coatomer recruitment during budding (Ktistakis *et al.*, 1996; reviewed in: Ktistakis, 1998). Several experimental data exist which provide strong evidence for a direct role of ARF1 as assembly factor of the COPI coat: (i) ARF1 has been shown to be present in stoichiometric amounts to coatomer (Stamnes *et al.*, 1998; Serafini *et al.*, 1991a); (ii) a direct interaction of ARF1-GTP with coatomer has been demonstrated by site-specific photocrosslinking. ARF1 with a photoreactive phenylalanine at position 82 was exclusively crosslinked to BG-COP when bound to Golgi membranes. This interaction seems to persist during budding because it is also found in isolated vesicles (Zhao *et al.*, 1997). Interestingly, an ARF1 mutant carrying the photoreactive site at position 46 in the putative effector loop (Amor *et al.*, 1994) interacts with β -COP, and in addition with γ -COP (Zhao *et al.*, 1999); (iii) a subcomplex of β - and δ -COP is able to bind to Golgi membranes in an ARF1 and GTP-dependent manner (Pavel *et al.*, 1998), and (iv) coatomer has been shown to serve as an effector for efficient hydrolysis of GTP bound to ARF1 (Goldberg, 1999) (see 3.2.1). The model that subunits of coatomer might bind to the open effector site in the ARF1-ARFGAP complex is in agreement with the positions in ARF of amino acids residues shown to interact with coatomer (see above).

Another interaction predicted for COPI assembly would be binding of the coat components to proteins present in the target membrane. Whereas no such interaction is presently established for ARF1, coatomer-membrane interaction has been shown to be mediated via cytoplasmic domains of members of the p24 family (see 3.5.1). Using a photocrosslinking approach, the γ -subunit of coatomer has been identified as the direct binding partner of the cytoplasmic tail peptide of p23 (Harter and Wieland, 1998). Thus, a bivalent interaction of coatomer with ARF1-GTP via a β -, γ -COP interface, and with p23 via γ -COP is likely to represent the molecular basis for COPI assembly (Figure 2).

FIGURE 2. Model for a bivalent interaction of coatomer with membrane-bound ARF1-GTP and with the cytoplasmic domain of a p23 tetramer. Interaction with ARF1 occurs via a β - γ -COP interface, and binding to p23 is mediated via γ -COP.



4.2. Reconstitution of Coated Vesicles from Chemically Defined Liposomes

In order to define the minimal requirements for the formation of COP-coated vesicles, their budding was reconstituted from chemically defined liposomes.

Using this reductionist system, it was shown that all that is needed to form a COPI-coated vesicle are the cytosolic proteins ARF and coatomer and the cytoplasmic domains of coat/cargo receptors (p24 family) emanating from the bilayer surface (Bremser *et al.*, 1999). Liposome-derived budding requires the presence of GTP and a temperature of 37°C similar to what is observed for COPI vesicle formation from biological membranes (Ostermann *et al.*, 1993). This budding reaction was shown to be completely independent of the lipid composition of donor liposomes provided that the cytoplasmic tail of p23 (or another p24 family member) was present. As in this reconstitution only the cytoplasmic domains of the p24 proteins are essential, an interaction of p24 luminal domains with cargo proteins does not seem to be necessary for coat assembly, consistent with earlier observations (Orci *et al.*, 1986). COPI- (and COPII-) coated vesicle formation from chemically defined liposomes without the need for any membrane protein was reported depending on special lipid compositions (Matsuoka *et al.*, 1998; Spang *et al.*, 1998). However, this budding reaction was independent on temperature and required a non-physiological composition of phospholipids as well as the presence of a non-hydrolyzable analogue of GTP. Thus, this reconstitution is likely to represent a partial event in budding, bypassing other steps.

Taken together, reconstitution of COPI vesicle budding from artificial lipid bilayers confirm that the molecular basis for assembly of a COPI vesicle is provided by a bivalent interaction of coatomer to form a trimeric

complex between the membrane-bound ARF1-GTP and the cytoplasmic tails of p24 family members (Figure 2).

4.3. Polymerization of Coatomer and COPI Bud Formation

With the knowledge of the minimal requirements for COPI budding, a still open question is: how does recruitment of a coat drive formation of a curvature in a membrane?

An answer to this basic question may come from *in vitro* binding studies with coatomer and the cytoplasmic tail peptide of p23. The results of these studies suggest that a tetramer of p23 induces specific polymerization of the coatomer complex (Reinhard *et al.*, 1999). Polymerization of coatomer is accompanied by a conformational change of the complex resulting in an increased susceptibility to protease of its γ -subunit (the direct binding partner of p24 family cytoplasmic domains). This polymerization on the surface of a membrane might be the driving force to shape a membrane into a coated bud.

Strikingly, the conformational change and polymerization of coatomer is specifically induced by some p24 cytoplasmic domains, but not by a peptide with a characteristic KKXX ER-retrieval motif that binds coatomer with similar efficiency and via the same subunit as p23 (see 3.4 and 3.5.1). Thus, the two classes of cytoplasmic domains of coatomer binding proteins seem to have two distinct and different functions: interaction of a retrieval motif might serve the sorting of membrane cargo into retrograde COPI vesicles. p23 and p24, however, represent part of the machinery of a COPI vesicle involved in vesicle budding (Figure 3).

5. MECHANISM OF VESICLE FUSION

Delivery of cargo to its destination by a transport vesicle critically depends on the accurate and specific membrane recognition and fusion. This involves membrane machinery for specific pairing of the membranes to be fused and cytosolic machinery that is involved in the timing and regulation of the fusion event. During the past few years, many components involved in membrane fusion have been identified at the molecular level. However, their precise function and the order of events are a matter of current debate. In the following section we will summarize the current view over heterotypic membrane fusion, e.g. fusion between a vesicle membrane and a target membrane and the components involved (Figure 4).

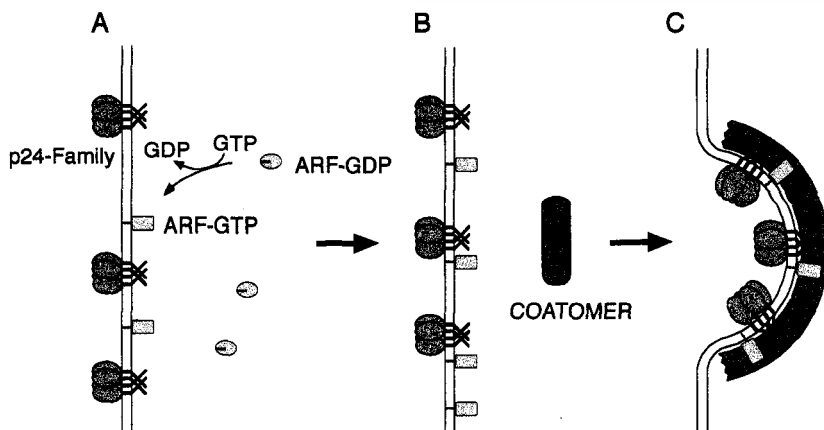


FIGURE 3. Model for the formation of a COPI-coated bud. **A**, Coated vesicle formation is initiated by the recruitment to the Golgi membrane of ARF1 from the cytoplasm. This requires exchange of GDP for GTP, a reaction catalyzed by a nucleotide exchange factor. **B**, Subsequent binding of coatomer is mediated by its interaction with the cytoplasmic domain of a tetramer of p24 family members, e.g. p23. **C**, Interaction of coatomer with the p24 tetramer induces a conformational change and polymerization of the complex that shapes the membrane into a coated bud.

5.1. SNARE Proteins

It is generally accepted that specificity in membrane recognition relies on the pairing of SNARE proteins, a family of soluble NSF attachment protein (SNAP) receptors (Figure 4B). The concept of a pair-wise recognition of SNARE proteins that reside in opposite membranes—the SNARE hypothesis—was first formulated by Rothman and colleagues for vesicle docking at the synapse (Soellner *et al.*, 1993b; reviewed in: Rothman and Warren, 1994). This hypothesis was originally based on the discovery of a core complex consisting of v(esicle)-SNAREs and t(arget)-SNAREs that have been identified as constituents of synaptic vesicles and the pre-synaptic plasma membrane, respectively (Soellner *et al.*, 1993b). In the nerve terminal, the core complex consists of vesicle-associated proteins, VAMP/syntaxin, from the synaptic vesicle, and of target membrane-associated proteins, syntaxin and SNAP-25 (which is unrelated to α -, β -, γ -SNAP) from the plasma membrane (Scheller, 1995).

Many v- and t-SNAREs have been characterized now in plants, yeast and mammals and it has been shown that they belong to gene families each of which comprising various numbers of isoforms. Specific isoforms

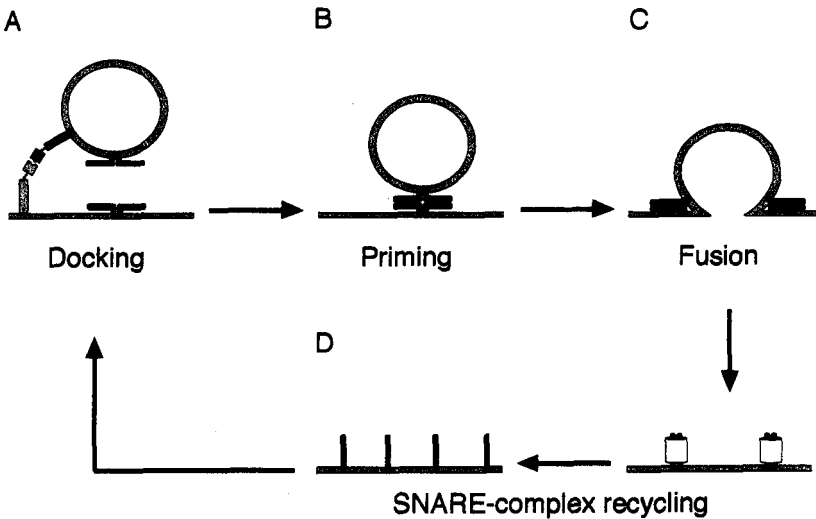


FIGURE 4. Model for heterotypic membrane fusion of a transport vesicle with its target membrane. **A**, The tethering of a vesicle to its target membrane involves interactions between vesicle-associated and target membrane-associated proteins. **B**, Correct docking of the vesicle allows specific pairing of SNAREs that reside in opposite membranes and primes the membranes for fusion. **C**, Assembly of SNAREs into tight complexes triggers fusion to occur. **D**, Disassembly is mediated by binding of cytosolic proteins to SNARE complexes, and a prerequisite for another round of vesicle fusion. For details and references see text.

localize to various organelles of the secretory pathway and it has been suggested that the heterogeneity in secretion may be defined by the large number of possible combinations of the various isoforms of SNARE proteins (Linial, 1997).

SNARE proteins can be divided into two classes: members of one class (family members of VAMP/synaptobrevin and the syntaxins) are anchored in the membrane via a C-terminal transmembrane anchor and expose most of their N-terminal domains into the cytosol, whereas members of the other class (SNAP-25 family) are anchored in the membrane by fatty acid acylation of one or more cysteine residues present in the middle of the molecule (reviewed in: Linial, 1997). A combination of electron microscopic (Hanson *et al.*, 1997), spectroscopic (Lin and Scheller, 1997), and X-ray crystallographic data (Fernandez *et al.*, 1998; Sutton *et al.*, 1998) has shown that SNAREs are able to form helices with potential for coiled-coil formation (reviewed in: Skehel and Wiley, 1998). These studies revealed that the cytoplasmic portions of SNAREs form a rod-shaped complex that is a coiled-coil of four α -helices. The four helices are arranged in a parallel manner,

one helix contributed by the v-SNARE and three by the t-SNAREs. Formation of this complex involves an increase in the content of α -helices as shown by CD spectroscopy. This change from a relatively unstructured, toxin protease-sensitive into a highly ordered state correlates with thermic stability and SDS resistance (Hayashi *et al.*, 1994). It is interesting to note that similar rod-shaped α -helical bundles have also been identified in the ectodomains of various viral fusion proteins (Weissenhorn *et al.*, 1998; Weissenhorn *et al.*, 1997; Fass *et al.*, 1996). Thus, assembly of otherwise unrelated proteins into similar molecular arrangement might serve to bring the membranes to be fused into close contact.

While it is widely accepted that membrane fusion within a cell between functionally different compartments involves members of the three families of SNARE proteins described above, it has been a matter of debate whether these proteins might also be sufficient for specific pairing of vesicles and subsequent bilayer fusion. In order to test this possibility cognate v- and t-SNAREs were reconstituted into separate lipid bilayer vesicles (Weber *et al.*, 1998). All that is needed for these vesicles to fuse is a rise in temperature to 37°C, while at lower temperature docked unfused intermediates seem to accumulate. Based on this data, it is suggested, that SNAREs provide the minimal machinery for pairing and fusion of membranes (Figure 4B–C).

5.2. NSF

The identification of the first protein involved in membrane fusion within the secretory pathway goes back to the 1980s when intracellular transport of VSV-G protein was reconstituted in a cell free system (Balch *et al.*, 1984a). Using isolated Golgi membranes, it was shown that VSV-G protein is packaged in coated vesicles, and that fusion of these vesicles is blocked by the cysteine-alkylating agent, N-ethylmaleimide (NEM) (Malhotra *et al.*, 1988). This led to the identification of NEM sensitive factor, or NSF (Block *et al.*, 1988; Malhotra *et al.*, 1988). A role in membrane fusion was also confirmed by the finding that mutants of Sec18p, the homologue of NSF in yeast, accumulate docked transport vesicles unable to fuse with their target membranes (Wilson *et al.*, 1989). It is now apparent that NSF plays a crucial role in all stages of the secretory pathway from the ER to the plasma membrane as well as during endocytosis. However the mechanistic details underlying NSF action are still under debate.

The structural and functional organization of NSF is well characterized: NSF is a cylindrical oligomeric ATPase with a three domain organization (Hanson *et al.*, 1997; Whiteheart *et al.*, 1994). The N-terminal domain,

designated N, binds to soluble NSF attachment proteins (SNAPs), a prerequisite for the interaction of NSF with Golgi membranes, and with SNAREs (Whiteheart *et al.*, 1993). The C-terminal domains, designated D1 and D2, are involved in ATP hydrolysis (D1) and ATP-dependent oligomerization (D2), both functions being essential for NSF's role in promoting membrane fusion (Whiteheart *et al.*, 1994).

In the original view about NSF function, it was suggested that NSF and SNAP would bind to a pair of cognate SNAREs that bridge the membranes to be fused, and that ATP hydrolysis by NSF would trigger membrane fusion (Whiteheart and Kubalek, 1995; Soellner *et al.*, 1993a). According to this concept NSF would function as a general fusion factor that would link membrane fusion with disassembly of the SNARE complex. This was based on the discovery of NSF and SNAP as constituents of the so-called 20s fusion particle that is formed in the presence of ATPγS or Mg-chelating agents, and SNAP receptors (Soellner *et al.*, 1993b). However, more recent data suggest that NSF is required for steps preceding the fusion event rather than for actual bilayer fusion (Mayer *et al.*, 1996). The role of NSF is suggested to lie in disassembly of SNARE complexes in order to make monomeric SNAREs available for another round of vesicle docking and fusion (Figure 4D) (reviewed in: Nichols and Pelham, 1998).

5.3. Additional Proteins Involved in Vesicle Fusion

Although the *in vitro* reconstitution experiments discussed above have demonstrated that SNAREs are sufficient for vesicle docking and fusion, it is likely that in a living additional factors are required (reviewed in: Pfeffer, 1999). For example, vesicle fusion must be controlled temporarily. If SNAREs could pair at all times, these would lead to uncontrolled fusion of organelles. Furthermore the pairing of SNAREs must be controlled spatially. This is important because the localization of SNAREs is not restricted to specific membrane sites (Garcia *et al.*, 1995). In addition, it has been shown that a single v-SNARE is able to pair with two different t-SNAREs, i.e. may be involved in more than one fusion event (von Mollard *et al.*, 1997). Thus, proteins must exist that assure vesicle fusion at the right place and to the right time. In the following we will give an overview of candidates involved in the spatial and temporal control of vesicle targeting and fusion.

5.3.1. Targeting Proteins

The molecular interactions underlying the targeting and docking of a transport vesicle is best understood for intra-Golgi transport in mammalian

cells and for ER-to-Golgi transport in yeast. In mammals, a function in vesicle docking was attributed to p115, a peripheral Golgi membrane protein, which was originally identified as a factor needed for intra-Golgi transport (Waters *et al.*, 1992).

Similarly, the yeast homologue of p115, Uso1p, was shown to be essential for docking of ER-derived vesicles to Golgi membranes (Barlowe, 1997). Both p115 and Uso 1p are myosin-like molecules: elongated homodimers with two globular heads and an extended coiled-coil tail (Yamakawa *et al.*, 1996; Sapperstein *et al.*, 1995).

In mammals, the docking site on the Golgi membrane of p115 has been shown to be provided by the cis-Golgi matrix protein GM 130 (Nakamura *et al.*, 1997). Based on recent results using detergent extracts of Golgi membranes, it is suggested that p115 links vesicles to the Golgi membranes by serving as a bridge between GM 130 on Golgi membranes and giantin, a 400kD membrane protein of COPI vesicles (Soennichsen *et al.*, 1998). The idea that p115, GM 130, and possibly giantin form strings of fibres that link vesicles to their target membrane is consistent with observation by EM studies (Orci *et al.*, 1998) (Figure 4A).

In yeast, a possible target for Uso 1p could be TRAPP (for transport protein particle), a high molecular weight protein complex that consists of about ten polypeptides (Sacher *et al.*, 1998). However, such a function for TRAPP remains to be demonstrated.

Another system that might be involved in vesicle docking is the exocyst, a protein complex that localizes to the site of secretion in budding yeast (Terbush *et al.*, 1996). It has been suggested that the exocyst complex may establish a specialized site for exocytosis and mediate the docking of transport vesicles to this site (Guo *et al.*, 1999). However, a role of the mammalian complex in vesicle docking remains to be reported.

5.3.2. Modulators of SNARE Action

Members of two protein families have been shown to be involved in intracellular transport by their modulation of SNARE complex formation. One family comprises the SEC1 gene products and the other the Ras-related Rab/Npt proteins. Members of both families have been localized to various intracellular compartments and transport intermediates, and are therefore likely to regulate transport at various steps. However, their exact roles and sites of action remain to be clarified.

Members of the Sec1 family are peripheral proteins that directly bind to the N-terminal region of syntaxins. Various members of this family in

yeast (Aalto *et al.*, 1993), *Drosophila* (Salzberg *et al.*, 1993), and mammals (Pevsner *et al.*, 1994) have been shown to prevent formation of the core complex of SNAREs by binding to syntaxin. This led to a model according to which Sec1 proteins serve as negative regulators of SNARE function.

Members of the Rab/Ypt1 family are believed to coordinate spatially and temporarily the events underlying the targeting and fusion of transport vesicles (reviewed in: Novick and Zerial, 1997). The Rab family member, Ypt1p, has been suggested to be crucial for the formation of a v-/t-SNARE complex required for ER-to-Golgi transport in yeast (Sogaard *et al.*, 1994). For some mammalian Rab GTPases, an interaction with motor proteins is suggested (Echard *et al.*, 1998; Peranen *et al.*, 1996). This led to a model for vesicle trafficking in which vesicles are linked to motor proteins via Rab GTPase, thus translocating a vesicle to its correct docking site.

6. PERSPECTIVES

During the last few years considerable progress was made towards the understanding of the mechanisms underlying transport of material within the secretory pathway. The basic components involved in the formation, targeting and fusion of major transport intermediates seem to have been identified. However, several new findings also taught us that regulation of intracellular trafficking might be more complex than anticipated, and that our understanding about this issue is far from complete. Questions that now need to be addressed include: (i) to elucidate the mechanisms by which cargo is sorted into a COP-coated vesicle. In this respect the roles played by members of the p24 family and by ARF1 are of particular interest; (ii) to identify the factors that determine the direction of a COPI vesicle (this might well be linked to the role of the various p24 members); (iii) to understand the mechanism by which coatamer catalyzes GTP hydrolysis on ARF1-ARFGAP; (iv) to investigate whether a conformational change of coat proteins induced by binding to their receptors represents a general principle to polymerize the coat and thus to shape a membrane into a bud; (v) to investigate the sorting of lipids during vesicle formation, and (vi) to identify additional factors that are required for specific vesicle targeting and fusion.

ACKNOWLEDGMENTS. We thank Maria Isabel Geli, J. Bernd Helms, Andreas Schwarz, and Dominik Wegmann for critical comments on the manuscript. The work in the lab of the authors is supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 352).

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Chapter 2

Neurotoxins as Tools in Dissecting the Exocytic Machinery

Michal Linial

1. INTRODUCTION

1.1. The Core of the Exocytic Machinery—the SNAREs

Membrane fusion takes place in all cell types in processes as basic as the cell surface's growth up to regulated secretion found in nerve terminals. At any given time a constant flow of membranes in the form of vesicles is on transit between subcellular Compartments. Fusing and detaching of those vesicles is the mode through which cargo is transferred between different compartments or secreted from the cell surface membrane. Despite this constant exchange of membranes between compartments, the identity of each remains stable and the directionality of the process is maintained. This flow, known as vesicle trafficking relies on precise recognition between donor and acceptor membranes. Currently, we have a rather detailed description of the proteins that are involved in such recognition and eventually in fusion. In exploring such recognition and fusion events

MICHAL LINIAL Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, 91904, Israel.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

both *in vitro* and *in vivo*, a few dozen proteins were presented. Comprehensive surveys of the exocytic cycle and the molecules involved in vesicle trafficking are found in several review articles (Bennett, 1994; Jahn and Sudhof, 1994; Linial and Parnas, 1996; Pevsner and Scheller, 1994; Sudhof, 1995). Here, we limit our discussion to the SNAREs and their immediate associated proteins. The SNAREs were initially identified as membrane receptors which bind specifically to a cytosolic fusion complex composed of N-ethylmaleimide Sensitive Fusion protein (NSF) and Soluble NSF Attachment Proteins (SNAPs) (Sollner *et al.*, 1993b). The SNAREs consist of VAMP/synaptobrevin from the vesicle membrane and syntaxin and SNAP-25 from the plasma membrane. Immediately following the discovery of neuronal SNAREs, it was shown that several secretory yeast mutants are homologous to the SNAREs (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). Searching the genetic databases and expanding the search for other organisms revealed the existence of a high number of SNAREs' isoforms that belong to highly conserved protein families. For discussion see (Linial and Parnas, 1996).

The discovery of homologous proteins from yeast to neurons validated the unified view for all modes of secretion as stated by the SNARE hypothesis (Sollner *et al.*, 1993a). According to this view, vesicle targeting and fusion, as well as specificity, are governed by the interactions between vesicle-associated SNAREs (v-SNAREs, e.g., VAMP/synaptobrevin) and target membrane-associated SNAREs (t-SNAREs, e.g., syntaxin/HPC-1 and SNAP-25). These three SNARE prototypes are membranous proteins (Table 1) with distinct sub-domains that participate in protein-protein interactions (Fasshauer *et al.*, 1998; Lin and Scheller, 1997). Despite the large body of data on the SNARE proteins, the dynamic scheme according to which the association of the SNAREs drives the fusion event is still unknown (Gotte and von Mollard, 1998; Hanson *et al.*, 1997). What is also unclear is if the structural and biophysical details of the SNAREs and their lipid environment are sufficient to explain the process of fusion (Weber *et al.*, 1998). In any event, current data show that SNAREs play a central role in the fusion process by providing very close apposition of donor and acceptor membranes. This role for SNAREs is shared from neurons to yeast (Nichols and Pelham, 1998).

1.2. Direct Associates of SNAREs

The SNAREs make up the minimal core of the exocytic apparatus. The assembly of the SNAREs in a complex is essential for steps of membrane apposition preceding fusion (Edwardson, 1998). Consequently, synaptic proteins that are direct associates of SNAREs may determine their acces-

Table 1
Mammalian SNAREs prototype

Protein prototype	Alternative name	Length (aa)	Membrane topology	Localization (as SNARE ^a)	Neurotoxin sensitivity
VAMP	synaptobrevin	118	TMD, type II	SV v-SNARE	TeNT BoNT/B/D/F/G
syntaxin	HPC-1	288	TMD, type II	PM (SV ^b) t-SNARE	BoNT/C1 α -Latrotoxin ^c
SNAP-25		206	palmitoylation anchor	PM (SV ^b) t SNARE	BoNT/A/E/C1 ^d

TMD, transmembrane domain. PM, plasma membrane. SV, synaptic vesicle.

^a membrane localization according to definitions of v- and t-SNARE (Rothman, 1994). ^b fraction of t-SNAREs localized to SV membranes, ^c possible interaction via α -latrotoxin receptor (Petrenko, 1993; Shoji-Kasai *et al.*, 1994). ^d overlapping specificity of BoTx1 to SNAP-25 (Foran *et al.*, 1996).

sibility to participating in the SNARE complex. Such proteins may play different roles on secretion as listed by the following categories:

- (I) As positive regulators, i.e., binding of the protein results in presentation and exposing of a binding site essential for SNARE complex formation.
- (II) As negative regulators, i.e., binding serves to shade or disrupt a specific binding site, thus interfering with the SNARE complex formation.
- (III) As modifying enzymes, i.e., the associated proteins may themselves carry enzymatic activity such as kinases and consequently they determine the SNAREs' phosphorylation levels (Hirling and Scheller, 1996; Nielander *et al.*, 1995). It was demonstrated that all SNAREs undergo multiple post-translational modifications (Nielander *et al.*, 1995; Shimazaki *et al.*, 1996).
- (IV) As sorting/trafficking carriers, i.e., the associated protein may serve to escort the SNAREs throughout the cell compartments, during vesicle biogenesis, through axonal transport, and finally in endocytosis and recycling.
- (V) As coincidence detectors, i.e., allowing the stimuli for exocytosis to be transduced to the formation and stabilization of the SNARE complex.

Some of the associated proteins may play multiple or even conflicting roles for the SNAREs, according to the specificity of the secretory system

(Table II). This is exemplified in the case of synaptotagmin I. It is an abundant synaptic vesicle protein that was proposed to be the Ca^{2+} -sensor of the exocytic machinery. It belongs to a large superfamily of C2 containing proteins (DeBello *et al.*, 1993; Popov and Poo, 1993) and in mammals belongs to a gene-family of about a dozen genes. Synaptotagmin I binds to syntaxin in a Ca^{2+} -dependent manner. It also binds to SNAP-25 and thus by itself, may be considered as a v-SNARE (Schiavo *et al.*, 1997). As a coincidence detector, synaptotagmin I contains binding sites for Ca^{2+} , inositol high polyphosphates, negatively charged phospholipids and phosphoinositols (Schiavo *et al.*, 1996). Its interaction with syntaxin is modulated by the level of such signaling molecules (Linial, 1997; Sudhof and Rizo, 1996). Indeed, binding of one or more of the signaling molecules to synaptotagmin (e.g., Ca^{2+} , inositol high polyphosphates) induces a global change in its structure (Davletov and Sudhof, 1994). As a trafficking carrier protein, synaptotagmin participates in the endocytic steps through its interaction to the adaptor protein AP2 (Zhang *et al.*, 1994). A list of major regulators of the SNAREs which act as direct associates is presented (Table 2).

1.3. Dynamic View of Secretion

A more dynamic view of the SNARE hypothesis suggests that NSF, which operates as a molecular switch via ATP hydrolysis, acts on the SNARE complex by interacting with a-SNAP. It is the ATP hydrolysis that drives the dissociation of the complex, releasing syntaxin, SNAP-25 and VAMP. Accumulating evidence suggests that an ATP dependent step is needed to disrupt the very stable complex of the SNAREs. If indeed the stable complex is a byproduct of the fusion event, then following ATP-dependent disruption of the complex, individual SNAREs will be free to engage in an additional cycle of fruitful interaction (Zheng and Bobich, 1998a). It may be a rate limiting step, and thus the ATP activation refers to an essential priming of the SNARE. See examples in (Haynes *et al.*, 1998; Lawrence *et al.*, 1994). Alternatively, it may not be needed for the actual exocytic process but rather for the balance of exo-endocytosis levels. In models such as brain synaptosomes, it seems that the ATP step is not essential for the Ca^{2+} -depolarization evoked release but rather for unwrapping the stable SNARE complex (denoted as the heat sensitive/SDS-resistant complex (Hayashi *et al.*, 1994) to its preassembled stage (Bennett and Scheller, 1994). However, as discussed above (Table 2) the SNAREs interact transiently with additional proteins such as synaptotagmin, synaptophysin and nSec-1/munc-18, complexin and others (Bennett, 1995; Hata *et al.*, 1993; Pevsner *et al.*, 1994; Scheller, 1995; Sollner *et al.*, 1993b). The sequential order of events in secretion is still not resolved (Edwardson,

Table 2
SNAREs' associated proteins

Topology	Protein name	Localization	Association to	Proposed role	Selected references
Multi TMD	synaptophysin	SV	VAMP	II	(Calakos and Scheller, 1994)
	N-type Ca^{2+} channel	PM	syntaxin, SNAP-25	I, II, V	(El Far <i>et al.</i> , 1995)
	P/Q-type Ca^{2+} channel	PM	syntaxin, SNAP-25	I, II, V	(Martin <i>et al.</i> , 1996)
	CIRL / Latrophilin	PM	syntaxin	I, II, V	(Krasnoperov <i>et al.</i> , 1997)
Single TMD	synaptotagmin	SV	syntaxin	I, II, IV, V	(Chapman <i>et al.</i> , 1995)
			SNAP-25	I, II, IV, V	(Schiavo <i>et al.</i> , 1997)
	VAP-33	PM	VAMP	I	(Weir <i>et al.</i> , 1998)
	Rab3	SV	SNAREs	I, II	(Horikawa <i>et al.</i> , 1993)
Lipid anchorage	Go	PM	syntaxin	II, V	(Linial, unpublished)
	Hrs-2	PM ?	SNAP 25	I, III	(Bean <i>et al.</i> , 1997)
Direct association	GAP-43	PM, SV	syntaxin	I	(Hens <i>et al.</i> , 1993)
	complexin	PM, SV	syntaxin, SNAREs	I	(McMahon <i>et al.</i> , 1995)
	n-Sec1 / munc-18	PM, SV	syntaxin	I, II	(Halachmi and Lev, 1996)
	α/γ SNAP	PM, Cyt	SNAREs	I	(Sollner <i>et al.</i> , 1993b)
	mAChR	PM	SNAREs	II, V	(Linial <i>et al.</i> , 1997)
	tomosyn	PM ?	syntaxin, SNAP-25	I	(Fujita <i>et al.</i> , 1998)
	calmodulin	PM, SV	via synaptotagmin	V	(Fournier <i>et al.</i> , 1989)
Indirect association	CAM KII	sv	via synaptotagmin, VAMP	III, V	(Nielander <i>et al.</i> , 1995)
	p60src	SV	via synaptophysin	III	(Pang <i>et al.</i> , 1988)
	AP2	PM	via synaptotagmin	IV	(Li <i>et al.</i> , 1995)
	NSF	PM, Cyt	via A/GSNAP	I, III	(Banerjee <i>et al.</i> , 1996)

The proposed roles (I–V) are described in the text. PM, plasma membrane; SV, synaptic vesicle; Cyt, cytosol.

1998; Jahn and Hanson, 1998; Zheng and Bobich, 1998b). Specifically, the exact stage along the secretory cycle that responds to depolarization or to Ca^{2+} -ions is still obscure. Synaptotagmin is the prime candidate for carrying the Ca^{2+} sensitivity and the coupling of Ca^{2+} stimuli to the release process. A protein complex of 7s composed of SNAREs and synaptotagmin is believed to represent an intermediate complex along the cycle of the synaptic vesicle life cycle. In such complexes the SNAREs are already associated, but the association is still very labile and probably reversible.

Following the introduction to the molecular view of secretion, we may introduce the neurotoxins that became unreplaceable tools in describing the chain of events leading to exocytosis. In the first part we will introduce the two groups of neurotoxins that interfere with secretion and neurotransmitter release. These toxins include two structurally and functionally distinct groups, the bacterial clostridial toxins, represented by tetanus toxin (TeNT) and toxins from the black spider venom, represented by α -latrotoxin. The clostridial toxins block neurotransmitter release while the latrotoxins induce massive and uncontrolled release. This is briefly summarized in Table I. The mode of action of bacterial clostridial toxins was recently unveiled and it will be further discussed.

2. LATROTOXIN AND RELATED TOXINS

2.1. Biology of the Toxins—Cell Recognition

α -Latrotoxin is the active component from the black widow spider venom. The effect of α -latrotoxin on neurotransmitter release was documented in neuronal, neuroendocrine, glial cells as well as several cell lines (Ceccarelli *et al.*, 1988; Hurlbut *et al.*, 1994; Meldolesi, 1982). The physiological effects were reported in the neuromuscular junction (NMJ) of the frog, mammals' CNS and PNS and in the *Torpedo* electric organ (Linial *et al.*, 1995). In all cases, an induced massive vesicle release is recorded (Grishin, 1998; Rosenthal *et al.*, 1990). Observations over the last two decades argue for a dual mechanism of the toxin. For example, in frog NMJ and under physiological solutions (high Ca^{2+}), binding of α -latrotoxin to its receptor induces the following phenomena: membrane depolarization, stimulation of Ca^{2+} influx, an increase in the cytoplasmic free Ca^{2+} concentration and stimulation of phosphoinositide breakdown. Only some of these effects are recorded in a Ca^{2+} free medium (Scheer *et al.*, 1986). It is intriguing that the toxin's multiple mechanisms lead to a massive unsynchronized vesicular release (Rosenthal *et al.*, 1990) both of classical neurotransmitters and of neuropeptides (Bittner *et al.*, 1998; Lang *et al.*, 1998; Liu and Misler,

1998). α -Latrotoxin also forms non-selective cation channels in artificial membranes prepared from neuronal or neuroendocrine cells (Rosenthal *et al.*, 1990). Formation of such channels depends on the presence of appropriate receptor molecules. Such channels are not formed in membranes prepared from non-neuronal sources (Filippov *et al.*, 1990). It is suggested that in neurons, but not in other cells, channels form by association of toxin molecules and their insertion into the membrane after binding to specific receptors at active zones. Synchronization of channel openings in such channels is promoted by Ca^{2+} (Filippov *et al.*, 1994). Accordingly, it is the influx of Ca^{2+} through these open cation channels that induces synaptic vesicle fusion (Grishin *et al.*, 1993).

In an attempt to purify α -latrotoxin receptors from mammalian brain synaptosomes neurexin 1 α was identified as a potential Ca^{2+} -dependent receptor (Petrenko, 1993). Neurexin 1 α binds at high affinity to α -latrotoxin in the presence of Ca^{2+} ions (Davletov *et al.*, 1996; Geppert *et al.*, 1998). *In vitro* binding studies revealed a small domain in synaptotagmin's C-terminal as the high affinity binding site for neurexin 1 α (Hata *et al.*, 1993). From the Ca^{2+} -independent activity of the toxin, it was suggested that the toxin acts via a different, independent bypass mechanism. A Ca^{2+} -independent receptor of α -latrotoxin, named CIRL/latrophilin was recently studied (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997). This receptor is an orphan G-protein coupled receptor. While the downstream effects of CIRL/latrophilin are not yet known, the binding of α -latrotoxin may activate the corresponding G-protein cascade. This activity may regulate presynaptic ionic channels, e.g., Ca^{2+} or K^{+} channels. We will not discuss the different possible modes of action of the toxin as its actual targets are not yet known. See discussion in (Ichtchenko *et al.*, 1998; Linial, 1998).

2.2. Structure and Biochemical Properties

α -Latrotoxin is a 130 kDa protein with a rather complicated structural domain including many tandem ankyrin repeats (Kiyatkin *et al.*, 1995). A panel of monoclonal antibodies against different domains in α -latrotoxin revealed the distinct domains in charge of the toxin's different functions. These include high-affinity binding, Ca^{2+} uptake, neurotransmitter release and formation of cationic channels (Pashkov *et al.*, 1993). Recently, several recombinant α -latrotoxin variants were produced and their ability to bind to CIRL/latrophilin and to neurexin 1 α was evaluated (Ichtchenko *et al.*, 1998). The structure shared by α -latrotoxin and its related members is seen in Figure 1. The structural information is extracted from the similarity in domain structure between α - and δ -latroinsectotoxins and the vertebrate specific α -latrotoxin. The major domains are the signal peptide, a conserved

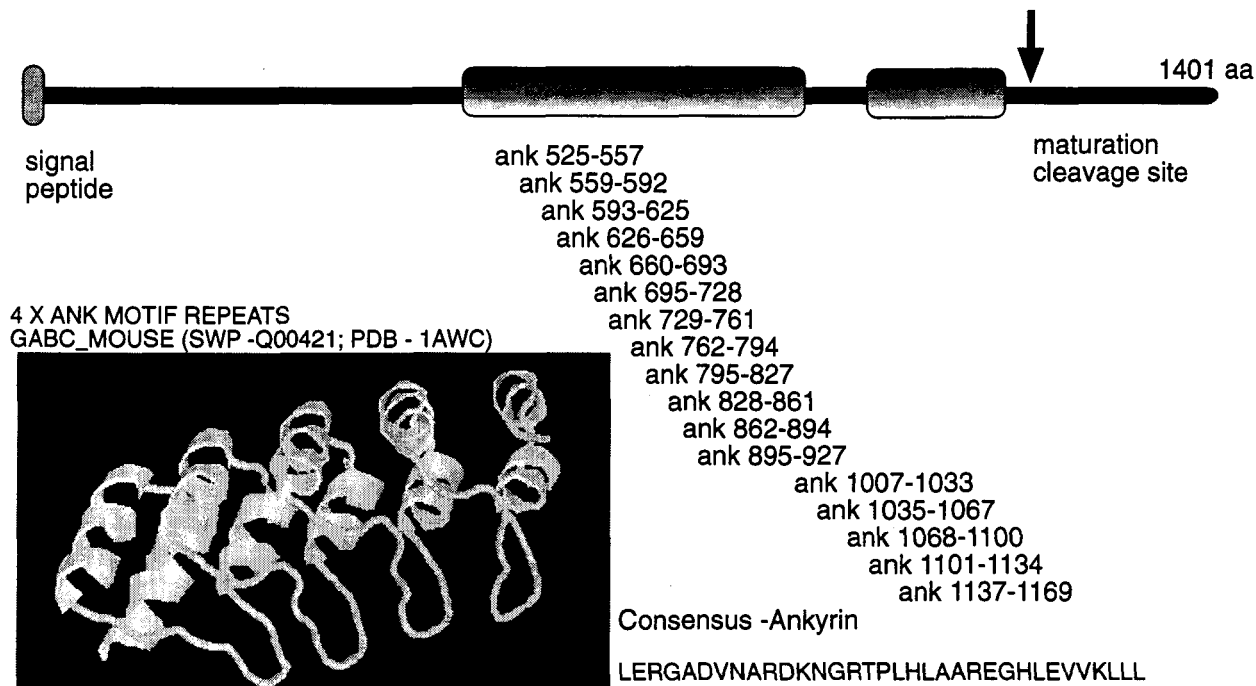


FIGURE 1. Schematic view of α -latrotoxin from black widow spider. The full length protein (1401 aa) contains a signal peptide and tandem ankyrin repeats. The repeats (ank) are marked according to their aa location. The functional toxin consists of the amino-terminal truncated fragment (the proteolytic site is marked by an arrow). Possible structure for α -latrotoxin is deduced from proteins containing multiple ankyrin repeats whose structures were resolved. The example shown is of the transcription factor gabc_mouse (PDB I.D. lawc) with its 4 ankyrin repeats. The 34aa sequence shown is a representative of an ankyrin domain prototype.

N-terminal domain that participates in receptor binding and in intramolecular stabilization. The major part of the protein consists of tandem ankyrin motifs. The repeats account for about 2/3 of the length of the functional molecules with 17–22 repeats (the variation in number depends on the stringency of the ankyrin domain definition). The carboxy-terminal of the protein is cleaved post translational to produce an active toxin. This structure is conserved in all latrotoxin family members. The mode of action of a-latrotoxin is not fully resolved and will not be further discussed.

3. CLOSTRIDIAL TOXINS

3.1. Biology of the Toxins—Cell Recognition and Activation

Unique proteins produced by the clostridium bacteria cause neuromuscular paralysis. Seven serotypes of botulinum neurotoxin (types A to G) and one tetanus toxin are responsible for the multiple syndromes of these toxins. While a common feature of all of these neurotoxins is the complete blockade of neurotransmission in the nerve cells, the differences in the pathology of the diseases are explained by the toxins' effect on different nerve cell types. While the tetanus toxin (TeNT) affects interneurons in the spinal cord, the botulinum toxin (BoNT) affects peripheral cholinergic transmission. These neurotoxins are the most potent natural toxins known to date, exerting their effect already at the subfemtomolar levels (Montecucco and Schiavo, 1995).

The biology, pathology and clinical aspects of TeNT and BoNTs were extensively discussed in the literature. The toxins require a very specialized mechanism for their activation. The potential of the toxin to block neurotransmission is exhibited only following several distinct cellular steps that activate the toxin and bring it to its final destination (Figure 2).

Presynaptic membrane recognition: The toxins bind to the presynaptic membrane in a highly specific and selective manner. Despite enormous efforts to identify the putative membrane receptors, it is still unclear who are the receptor molecules or even what is their biochemical nature (Lazarovici, 1990). Evidently specific polysialogangliosides are involved in the initial binding of clostridial toxins to synaptic membranes, but it is insufficient to account for the binding specificity of each clostridial toxin serotype. It seems that the toxins' specificity is determined by a combined interaction (e.g., lipid and a membrane protein). Proteins enriched in the synapse are the best candidates as acceptor molecules. Specifically, the synaptic vesicle protein, synaptotagmin, may participate in BoNT/B recognition and internalization (Kozaki *et al.*, 1998).

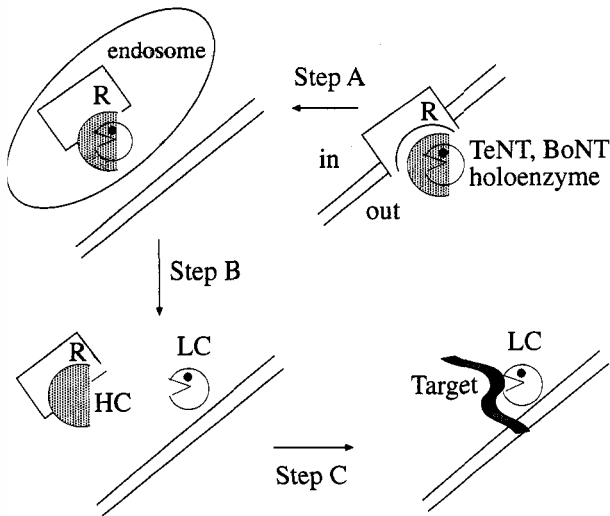


FIGURE 2. Schematic steps in the intoxication process by BoNTs and TeNT. Step A—Following binding of the toxin to its receptor from the extracellular face as an holoenzyme, the receptor-toxin complex undergoes endocytosis and internalization into an acidic compartment. Step B—At this stage, the toxin is being activated and the light chain (LC) and heavy chain (HC) are separated. The active component (LC) is translocated to the cytoplasm. Step C—The active LC recognizes its target and cleaves a unique peptide bond in the protein (for further details see text).

Internalization and targeting: Shortly after the toxin is bound to the membrane, it is internalized via sub-organelles that are reminiscent of the endosomal compartment. The formation of small endosome-like vesicles in the nerve cell is an active and energy dependent process. After internalization, TeNT and BoNTs are targeted to different cellular routes. TeNT moves retrogradely from a peripheral nerve, along its axon to and across the synapse of the interneurons in the spinal cord. In contrast, BoNTs are being activated in the same neuromuscular synapse that they were initially bound to. The function of BoNTs and TeNT converges again after the toxins reach their cellular targets. The low PH of the endosomal compartment activate the toxin by reducing the cysteine link holding the Light chain (LC) and the Heavy (HC) of the toxin.

Exposure of the toxins to the cytosol following activation: The process of toxin exposure requires active disruption of the endosomal-like compartment. In analogy to other bacterial toxins (e.g., diphtheria toxin), a low pH activation step is needed for the catalytic activity of the toxin. Once the

toxin is activated and targeted to the cytosol as an active peptide chain, it can reach its substrate. The substrate are one of the SNARE proteins (as detailed below).

The steps are schematically described in Figure 2. Still we do not have a complete view on the nature of the cell biological processes involved.

3.2. Structure and Biochemical Properties

All Clostridium toxins share a similar basic structure. The toxin precursor is a 150kDa polypeptide encoded by a single gene. Following specific cleavage, two polypeptides of 100 kDa (Heavy chain, HC) and 50 kDa (Light chain, LC) are formed and are joined by a disulfide bond. The toxin is functional only following exposure of the free LC to the nerve terminal milieu (Montecucco and Schiavo, 1995).

Sequence comparison among all clostridial neurotoxins shows that only the LC is conserved between the different toxins' subtypes. It is consistent with the view that the catalytic activity of the toxin resides on the LC while the HC determines the toxin's specificity and cellular targeting. A common motif found in the N-terminal of the LC among clostridial toxins is shared by various known zinc-dependent proteases (De Filippis *et al.*, 1995; Montecucco and Schiavo, 1993). Specifically, the shared amino acids are those which coordinate the Zn^{2+} atom in the protease active site. These amino acids are also shared among TeNT and all serotypes of BoNTs (Figure 3). Indeed, Zn^{2+} -chelating agents such as dipicolinic acid and captopril, inhibitors of Zn^{2+} -dependent peptidases, counteract the effect of the neurotoxin (Dayanithi *et al.*, 1994; Hohne *et al.*, 1994). The same effect was achieved by applying toxin that was mutated at the Zn^{2+} -binding domain. The mutated toxin did not have any effect even at high doses of the toxin (Li *et al.*, 1994).

These findings suggested a biological catalytic function for the clostridial toxins and induced a search for their natural targets. The first target of the clostridial toxin was identified as VAMP/synaptobrevin that was shown to be cleaved by TeNT (Table 1). VAMP/synaptobrevin is a major synaptic vesicle protein that was independently identified as a v-SNARE (Schiavo *et al.*, 1992; Yamasaki *et al.*, 1994a; Yamasaki *et al.*, 1994d). Shortly after this discovery, the entire picture was resolved and is schematically shown in Figure 4. In addition to TeNT, BoNT types B, D, F, and G also cleave VAMP/synaptobrevin (Figure 4). Similarly, additional synaptic proteins were identified as substrates for the different BoNTs (Blasi *et al.*, 1993a; Blasi *et al.*, 1993b). Specifically, BoNT/A and BoNT/E cleave SNAP-25 (Binz *et al.*, 1994) while BoNT/C1 cleave syntaxin/HPC-1 (Blasi *et al.*, 1993b). While the specificity was shown to be unique regarding the target

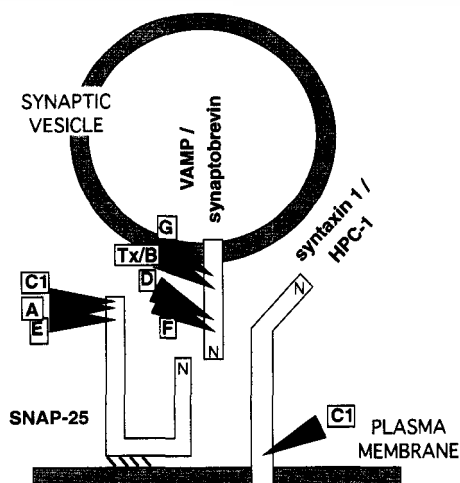


FIGURE 4. Specificity and cleavage sites of clostridial toxins. All three neuronal SNAREs are shown. VAMP/synaptobrevin is located on the synaptic vesicle membrane while syntaxin/HPC-1 and SNAP-25 are located on the plasma membrane. N marks the orientation of the amino termini of the proteins. The lipid association of SNAP-25 is representing the palmitates moieties. The arrowheads point to the cleavage sites for BoTN/G B, D and F (Yamasaki *et al.*, 1994a; Yamasaki *et al.*, 1994b) and TeNT in VAMP/synaptobrevin. The site of cleavage for BoNT/B and TeNT overlaps (Link *et al.*, 1992; Schiavo *et al.*, 1992). The cleavage sites of BoNT/E, A and C1 for SNAP-25 and of BoNT/C1 for syntaxin are shown (Blasi *et al.*, 1993a; Foran *et al.*, 1996). Note that the soluble fragments produced following cleavage by the toxins vary in their length. For example, following BoNT/C1 most of syntaxin molecule (aa 1–253) is truncated but only a very short fragment (aa 199–206) is cleaved following the cleavage of SNAP-25 by the same toxin.

protein and the exact cleavage site, it was confirmed that BoNT/C1 cleaves in addition to syntaxin also SNAP-25 (Foran *et al.*, 1996). In addition, TeNT and BoNT/B share the same peptide bond specificity for cleavage. The specificity of the different BoNTs is based on both recognition sites for the toxins and the availability of a precise amino acid sequence (Rossetto *et al.*, 1994; Washbourne *et al.*, 1998). An extreme example is the very close but distinct sites for BoNT/A, BoNT/E and BoNT/C1 on SNAP-25. The three sites are only few amino acids apart from each other.

In summary, it is the SNARE proteins (and only them) that have turned out to be targets for clostridial toxins (Link *et al.*, 1994; Montecucco and Schiavo, 1994; Schiavo *et al.*, 1994a; Schiavo *et al.*, 1994b). If indeed all the activities of the toxins rely on their ability to cleave their targets, a mutant toxin that lost its proteolytic activity should not show any

toxification while introducing intracellularly. Such experiments were carried out by mutating the toxins in the central histidine residues that are essential for the Zn^{2+} -dependent catalysis by the toxin (as in Figure 3). While in most studies such manipulation abolished the entire effect of the toxins (Yamasaki *et al.*, 1994c), in other cases a residual effect of the toxin was remained. The latter suggests an additional independent mode of action for the toxins, which is not attributed to their catalytic function (Ashton and Dolly, 1997; Gobbi *et al.*, 1996).

4. INVESTIGATING SECRETION WITH CLOSTRIDIAL TOXINS—THE METHODOLOGIES

4.1. *In vivo* —Genetic Approach

To study the synaptic function at the level of the organism itself a transgenic *Drosophila* was prepared by introducing a gene encoding TeNT-LC (Sweeney *et al.*, 1995). Toxin expression in embryonic neurons removed any detectable VAMP. The result was a complete elimination of evoked synaptic vesicle release. Interestingly, the effect was not observed on spontaneous release. In this *in vivo* experiments at the organism level, only the neuronal VAMP was cleaved by the toxin while the ubiquitous expressed protein remained resistant to TeNT. α -Latrotoxin stimulatory effects on secretion were not altered in TeNT expressing flies, while the release induced by hyperosmolarity was affected. This set of experiments provides a new tool to address fundamental questions on the molecular correspondence between spontaneous and evoked release and on the mechanisms that are activated by different mode of stimuli.

Another use of the clostridial toxins is in using organisms that were genetically manipulated. Experiments were performed on *Drosophila* (Littleton and Bellen, 1995), *C. elegans* (Nonet *et al.*, 1993; Nonet *et al.*, 1998) or mice following knockout of individual SNARE or other protein participating in neurotransmitter release (Broadie *et al.*, 1995; Brose, 1998; Littleton *et al.*, 1998). The action of the different clostridial toxins in the altered genetic background allows us to distinguish the roles of each SNARE in the morphology and physiology of the synapse throughout the complete life cycle of the organism.

Once the structure and the catalytic activity of the toxin were resolved, it became possible to apply the activated toxin directly to the cell of interest by microinjection. Such an application was successfully used in microinjection TeNT and BoNTs to identified cells in the buccal *Aplysia* ganglion (Cornille *et al.*, 1995; Poulain *et al.*, 1996) in squid giant synapse (Hunt *et*

al., 1994), in the Retzius synapse of the leech (Bruns *et al.*, 1997) and in the case of other large synapses suitable for injection (Stanley and Mirotznik, 1997). Such experiments allow us to combine electrophysiology and morphology studies. For example, microinjected of BoNT/C1 into the squid giant presynaptic terminal resulted in a complete block in transmission while the number or distribution of synaptic vesicles at the presynaptic active zone were not changed (O'Connor *et al.*, 1997). In such experiments the kinetics and the extent of inhibition by the toxins were determined.

4.2. *In vitro*—Tissues and Cells

The structure of clostridial toxins ensures that the toxins will exert their activity on cellular internal targets only following the activation and exposure to the cytoplasm. In cells, various methodologies were used. They are summarized in the following scheme (Figure 5).

Stable transfection. Introducing toxins to cells by stable transfection of the active toxin (the LC) is an alternative way of introducing the toxin to the cell milieu. In contrast to the microinjection procedure (see above), transfection is performed on the entire culture and the genetic material used for transfection may be genetically altered. For example, BoNT/A LC was transfected into AtT-20 cells. This manipulation resulted in a complete cleavage of SNAP-25. The transfected cells could not be induced to secrete ACTH upon stimulation (Aguado *et al.*, 1997). A more complex setting was performed in insulin-secreting HIT-T15 cells. There, BoNT/A was introduced into HIT-T15 cells following overexpressing SNAP-25. Transient expression of BoNT/A cleaved the endogenous as well as overexpressed SNAP-25 proteins and caused reduction in insulin secretion. This transfected system was also used to introduce the cell with SNAP-25 from which the C-terminal 9 amino acids had been deleted to mimic the effects of the toxin (see Figure 3). This modified SNAP-25 was as effective as the toxin at inhibiting secretion. The results of such manipulations are consistent with the view that the inhibitory action of the BoNT/A toxin is caused by the production of the cleavage product, which interferes with insulin granule fusion (Huang *et al.*, 1998). Morphological studies showed that upon treatment of the toxin or the transfected truncated SNAP-25 fragment, higher numbers of docked insulin granules are seen. This result suggests that block in secretion occurs following docking and that the toxin effect provided by a fragment of SNAP-25 that interact itself with the core complex in an unproductive manner.

Liposome transfection. An alternative mode of introducing active toxins to the cell interior is by liposome transfection of the active LC of the toxin with or without a peptide that overlaps the putative cleavage sites.

BoNT and TeNT - in vivo approaches
Primary cells, Cell lines and tissues

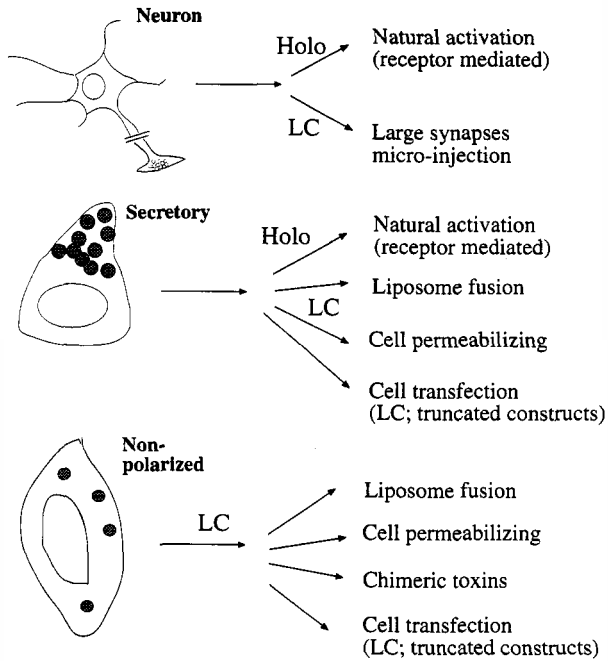


FIGURE 5. Schematic view on the methodologies used to study secretory systems by the use of TeNT and BoNTs. The model systems are (i) neuronal, (ii) polarized cells with regulated secretion pathway such as neuroendocrine cells or non-excitable cells such as the parotid acini and (iii) non-polarized cells which do not have a regulated secretory system. The mode of introducing the toxins intracellularly are indicated. LC and Holo are light chain and holoenzyme, respectively. For details see text.

While this is in principle similar to direct toxin injection, it is applicable to a large number of cells, tissues and culture cells. This methodology has a wider use as it is not sensitive to the physical size or shape of the cell of interest. This procedure was recently applied to a primary culture from cardiac myocytes (Kimura *et al.*, 1998). BoNT/A incorporated into liposomes was added to the culture medium and the effect of the uptake of the activated toxin mediated by the liposomes was monitored within a few days. Indeed, in this case, the beating rate of cardiac myocytes was markedly attenuated.

Permeabilized cells. The most widely used method for efficiently introducing the active form of the clostridial toxins is in permeabilized cells. This procedure includes exposure of the cells to streptolysin-O, α -toxin of *Staphylococcus aureus* or digitonin. This method of permeabilization leads to stable transmembrane channels that permit the introduction of small molecules into the cell but preserve the cellular structures and macromolecular contents. The permeabilization agents form small pores in the outer membrane without affecting the inner membranes (Ahnert-Hilger *et al.*, 1985). Once the cells are treated with these drugs, they are suitable for the introduction of antibodies, drugs, peptides and toxins and other impermeable nucleotide analogs. These procedures were performed to monitor secretion in chromaffin cells (Ahnert-Hilger *et al.*, 1992; Sanders and Habermann, 1992), PC12 cells (Ahnert-Hilger and Wiedenmann, 1994), pancreatic β -cells (Regazzi *et al.*, 1995), acinar cells (Stecher *et al.*, 1992), gonadotropes (Van der Merwe *et al.*, 1989) and more. The drawback of the procedure is the potential leakage of essential small components, such as ATP, GTP and small proteins. Still, when performed with highly purified reagents and under controlled conditions, the permeabilized cells maintain their functionality without substantial loss in their secretion capacity or in viability, as previously discussed (Van der Merwe *et al.*, 1989). In interpreting results after introducing clostridial toxins into permeabilized cells, it should be mentioned that dissipation of physiological membrane potential and fast dilution of small moieties are unavoidable. These are indeed crucial factors in control of neurotransmitter release (Jonas *et al.*, 1994; Linial *et al.*, 1997; Ohara-Imaizumi *et al.*, 1992).

The methods summarized above (see Figure 5) were developed to overcome the barrier of cell recognition receptors. This is essential in studying trafficking and secretion in non-neuronal system which lack a natural receptor for the toxins. An original way to bypass receptor recognition is by the construction of a chimeric toxin. In one such example, the lethal factor of anthrax toxin was fused to TeNT-LC. This protein was cytotoxic to many cell lines and tissues such as macrophage and CHO cells (Arora *et al.*, 1994). The toxin effect of the chimeric protein was dependent on the activity of the LC as a protease. The toxin within the cells cleaved cellubrevin (VAMP-3) and disrupted the intracellular membrane fusion mediated by a ubiquitous non-neuronal homologue of VAMP (see Figure 3).

4.3. *In vitro*—Overexpressed Proteins

Overexpressing SNAREs in various expressing systems was applied for the study of structure-function relationship. In this line of research, clostridial toxins became a very sensitive probe. Expression of the three

SNAREs either by *in vitro* translation systems or by expression in *E. coli* was used to explore their mutual interactions, their stability and their assembly properties. Over-expressed SNAREs were used initially to verify the exact cleavage site in the target protein in isolated situation (Binz *et al.*, 1994; Blasi *et al.*, 1993a; Schiavo *et al.*, 1993).

In vitro mixing of all three over-expressed SNAREs allowed formation of a stable SDS-resistant complex. Application of the clostridial toxins to the already assembled trimeric complex did not result in its disassembly. These results suggested that also *in vivo*, clostridial toxins will preferably attack the uncomplexed proteins (or at least not in their trimeric stable complex state). VAMP was also protected from proteolysis by TeNT under conditions that stabilize VAMP in the 7S protein complex (consists of SNAREs protein and synaptotagmin) (Sollner *et al.*, 1993b) or in the complex that also contains the NSF and SNAP proteins (also known as the 20s complex) (Hayashi *et al.*, 1994; Pellegrini *et al.*, 1994; Pellegrini *et al.*, 1995).

An *in vitro* simulation assay to reveal the effects of these neurotoxins on the SNAREs' assembly was performed. The results indicated that proteolysis of the proteins by different clostridial neurotoxins has inhibitory effects on the formation of SNARE complexes, but these effects depended on the type of toxin used. For example, the cleaved SNAP-25 by BoNT/A and BoNT/E shows reduced binding to VAMP and not to syntaxin (Chapman *et al.*, 1994; Hayashi *et al.*, 1995) but VAMP is able to participate in the 20s complex (Hayashi *et al.*, 1994; Otto *et al.*, 1995). Such simulations were also performed for the assembly of dimeric compositions (Hayashi *et al.*, 1994).

Recombinant v- and t-SNARE proteins were reconstituted into lipid bilayer vesicles. This combination induced the assembly of SNARE complexes and ultimately led to lipid mixing between separate liposomes. In these studies, the introduction of clostridial toxin abolished the formation of the essential SNARE complex, ensuring the role of the formation of a stable SNARE complex for fusion, even in such *in vitro*, artificial conditions (Weber *et al.*, 1998).

Most structural studies took advantage of the available expressing systems and the potential of following structural changes by combining SNARE molecules in a test tube. Proteins were expressed with or without a tag (i.e., hexa-histidine or GST-fusion proteins). The expressed proteins were purified by an affinity matrix and used for *in vitro* binding assays. Based on the knowledge that each of the clostridial toxins alter (to a lesser extend) the stability of the SNARE complex, a set of experiments using recombinant truncated SNAREs were performed. For example, the use of

overexpressed VAMP constructs mimicking the cleavage by TeNT or BoNT was tested. These studies pointed to the central domain of VAMP (the most conserved domain between different organisms, see Figure 6) as the domain interacting with the other two SNAREs. It is the same domain that was predicted to form an amphipathic α -helix (Chapman *et al.*, 1994). Similar analyses were performed for the other SNAREs. The purified expressed proteins were further used to allow detailed biophysical measurements. These studies revealed the extremely high melting temperature of the SNARE complex, as well as the overall orientation of the four helices involved (SNAP-25 contribute two helices) in the core complex (Fasshauer *et al.*, 1998; Poirier *et al.*, 1998). Based on the high resolution structural data of the SNARE core complex the effect of all seven clostridial serotype and TeNT on its stability can be predicted (Poirier *et al.*, 1998; Sutton *et al.*, 1998).

What is so special about these three synaptic proteins: SNAP-25, VAMP and syntaxin? Some of the answers may be attributed to their structure. All three proteins are membranous, either via a carboxy-terminal hydrophobic tail as in VAMP and syntaxin, or by a lipid modification as in SNAP-25 (Table 1). In all three proteins an amphipathic domain in the conserved region of the proteins is predicted. Moreover, syntaxin and SNAP-25 have a coiled-coil domain that is crucial for protein-protein interactions with each other and with additional key proteins. Therefore, it is not surprising that the protein-protein interactions of all three proteins are sensitive to any modification and alteration in their structure. Any change in the primary structure as a result of TeNT and BoNTs cleavage will interfere with the basic function of all three SNARE proteins, namely, the precise assembly with each other and with additional proteins for docking, fusion and recycling.

5. CLOSTRIDIAL TOXINS AS MOLECULAR PROBES FOR SECRETION

5.1. Probes for Evolutionary Conservation and Diversity

Once the targets of the clostridial toxins and their structural features were known, it became possible to apply the toxins as “genetic screening tools”. Clostridial toxins as site specific proteases provide probes to explore additional proteins that may serve as natural targets. As such, the toxins are sensitive tools for testing evolutionary conservation within the SNAREs and their homologues. Strong evidence for the importance of

the cleavage site specificity to the toxin's neuropathology is found in the case of VAMP. A single amino acid in the putative cleavage site for BoNT/B is different between rat and mouse VAMPs (Figure 6). This difference accounts for the apparent resistance of rat (but not mouse) to BoNT/B. The specificity toward different organisms is evident by comparing the specificity of binding sites between different organisms. The detailed example of the VAMP family ensures that in some of the sequences all TeNT and BoNT will act (e.g., syb1, syb2, syb3 in mouse) while other related sequences will be resistant to most clostridial toxins (syb-drome). Careful evaluation of the multiple alignment sequences within the SNARE family reveals that specific organisms will differ in respect to the sensitivity to one or another serotype of the clostridial toxins (Washbourne *et al.*, 1997). In Figure 6 an example of multiple alignment sequences of members related to VAMP/synaptobrevin are presented. Similar multiple alignment consensus is valid also to syntaxin (about 22 family members) and for SNAP-25 (about 8 family members). The combined knowledge about the specificity of the different clostridial toxins and the availability of large protein databases may be used for designing proteins with alternate susceptibility to clostridial toxins for basic research and clinical use.

5.2. Probes for Structural Specificity

BoNT/A and BoNT/E are metalloproteases with a unique specificity for SNAP-25 (Figure 3). This specificity of cleavage is determined by the site of cleavage itself but also by the presence of an appropriate recognition motif in the target (Rossetto *et al.*, 1994; Washbourne *et al.*, 1997). It has been suggested that this specificity is directed through the recognition of a nine residue sequence, termed SNARE motif (marked in boxes, Figure 3). This motif is common to the other two SNARE proteins: VAMP and syntaxin. Antibodies against this motif cross-react with VAMP, SNAP-25, and syntaxin and inhibit the proteolytic activity of the neurotoxins. The involvement of the SNARE motifs in SNAP-25 in the interaction with BoNT/A and BoNT/E was determined by following the kinetics of proteolysis of SNAP-25 mutants deleted of SNARE motifs. The results of such manipulations ensured that a single copy of the motif is sufficient for BoNT/A and BoNT/E to recognize SNAP-25. The toxins use the motif closest to the cleavage site, but, in its absence, distant copies of the motif are able to support proteolysis. This analysis shows the flexibility in recognizing a protein target for the BoNTs (Pellizzari *et al.*, 1996). These predictions are consistent with the sensitivity of SNAP-23 (a non-neuronal isoform of SNAP-25) to BoNT/E, but not BoNT/A, and the resistance of SNAP-25 isoforms from *Torpedo* and *Drosophila* to BoNTs. Searching the

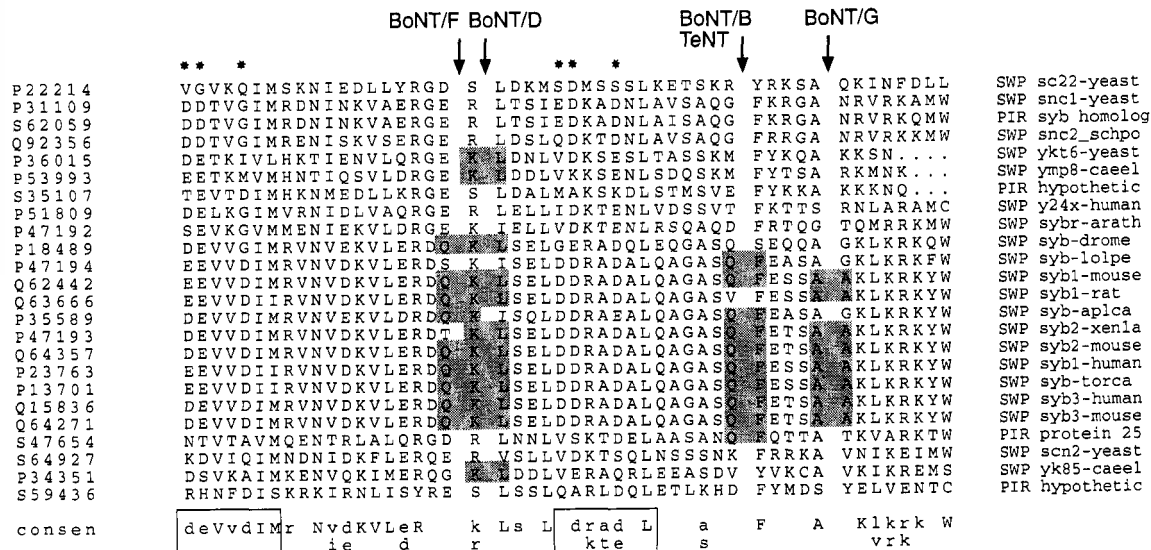


FIGURE 6. Multiple alignments of VAMP/synaptobrevin family members. The alignment was performed using PILEUP program (GCG Wisconsin Package). VAMP/synaptobrevin family members are derived from family classification using ProtoMap (<http://www.protomap.cs.huji.ac.il>). VAMP homologues representatives are from yeast, *C. elegans*, squid, *Drosophila*, Torpedo, frog and mammalian. The alignment covers the region in the proteins sufficient for cleavage by BoNTs and TeNT (aa 40–90 in syb2-rat). The peptide bond specificity for cleavage is depicted by the arrows and sequences containing the peptide bond for cleavage are shaded. Above the sequences are the two clusters of negatively charged residues which were implicated in the “SNARE motif” (Rossetto *et al.*, 1994; Washbourne *et al.*, 1997). The left columns lists the ID numbers from SWISSPROT (SWP) or PIR databases. A full description of the sequences is available in these databases. At the bottom line a simplified consensus (consen) sequence is constructed. In lower case and capital letters are aa which appear in at least 112 and 213 of the sequences, respectively. In cases that 2/3 of the residues are of the same group (e.g., negative, positive, polar, aromatic or hydrophobic) the two predominant aa are marked. The rectangles represent the minimal “SNARE motif” in VAMP family.

database allows detection of novel proteins that carry either the SNARE motif without the cleavage site specificity or vice versa.

5.3. Identifying New SNAREs, Their Associates and Their Roles

New SNAREs. Exocytotic events that were shown to be insensitive to the clostridial BoNTs and TeNT triggered the search for either SNARE-independent or SNARE-dependent events that are toxin-resistant. In the search for the molecular entities underlying toxin-resistant exocytosis, alternative SNAREs were identified. This includes syntaxin 3, SNAP-23, and a new TeNT insensitive VAMP isoform (TI-VAMP). This revealed a new set of SNAREs that are insensitive to clostridial neurotoxins. In epithelial cells, the two t-SNAREs (syntaxin 3 and SNAP-23) form SNARE complexes with TI-VAMP and cellubrevin (syb3, Figure 3). The combination of TI-VAMP with SNAP-23, and syntaxin 3 allows the formation of functional trimeric complex and they may underlie the observation of clostridial insensitive pathway for apical plasma membrane of epithelial cells (Galli *et al.*, 1998).

New SNAREs' associates. If indeed the formation of a stable SNARE complex precedes the fusion event, it is possible to alter the SNAREs by the clostridial toxins and to monitor the possible effect on their associated proteins. For example, in studying the G-protein regulation on the N-type Ca^{2+} channels, the effects of G-proteins on the channels were eliminated if syntaxin was cleaved with BoNT/C1. These findings indicate that the G-proteins are in (direct or indirect) association with the exocytic machinery (Stanley and Mirotnik, 1997). The role of the G-proteins as a modulator in the release apparatus through syntaxin has been recently confirmed in rat brain synaptosomes (Linial, unpublished results).

New roles for SNAREs. The clostridial toxins were used to probe new roles of SNAREs in addition to their role in exocytosis. Indeed, a new role for the SNAREs in development and in axonal growth was exposed. It was shown that t-SNAREs participate in membrane expansion at growth cones. BoNT/C1 treatment caused growth cone to collapse and inhibited axonal growth. Using video-enhanced microscopic it was shown that the vesicle-rich region in the growth cone was blocked. This demonstrated the central role for syntaxin and SNAP-25 (or both) in membrane expansion of growth cones (Igarashi *et al.*, 1996).

Another unexpected role of syntaxin was proposed for chromaffin cells (Bittner *et al.*, 1996). In syntaxin 1A transfected cells, the storage of the secretory pathway was induced. This observation was further confirmed by BoNT/C1 that partially reversed the effect of syntaxin on storage accumulation.

Still, the possibility that all secretory pathway eventually will turn to be SNARE-dependent (either containing toxin-sensitive or -insensitive SNAREs) is open. Such cases were proposed in systems outside the nervous system (Fiedler *et al.*, 1995; Ikonen *et al.*, 1995).

5.4. Studying the Diversity of Secretory Systems

The use of a battery of TeNT and BoNTs allows us to study to what extent SNAREs are involved in secretion and trafficking of many cell lines and systems. Studies in PC12 and in bovine adrenal chromaffin primary cells revealed a difference in term of the ATP dependency of the release. In both types of cells exocytosis can be triggered by μM amounts of Ca^{2+} , but chromaffin cells in addition require ATP. In permeabilized PC12 cells TeNT-LC blocks exocytosis in the absence of ATP similar to the effect obtained with permeabilized bovine adrenal chromaffin cells, in the presence of ATP. By contrast, BoNT/A-LC, which is highly potent in permeabilized bovine adrenal chromaffin cells, causes only a weak inhibition in PC12 cells. According to these studies it may be that while TeNT and BoNT/B probably block a common step during exocytosis from both PC12 cells and adrenal chromaffin cells, the accessibility of SNAP-25 to the action of BoNT/A is different between these two model systems.

The set of clostridial toxins and TeNT was applied in exocrine cells, platelets, mast cells, kidney cells, cholecystokinin-secreting cell lines and more. In the last case, the three neuronal SNAREs were detected. Introducing either of the TeNT or BoNTs to the streptolysin-O-permeabilized cells abolished Ca^{2+} -induced cholecystokinin secretion (Nemoz-Gaillard *et al.*, 1998). Similar results were also obtained in the exocrine pancreas and in the parotid cells (Fujita-Yoshigaki *et al.*, 1998; Gaisano *et al.*, 1994). In such systems, it is possible to evaluate the level of inhibition of secretion following treatment by the toxins. Partial inhibition is indicative for parallel secretory pathways or to the presence of unique SNAREs with different sensitivities to the toxins (Rossi *et al.*, 1997).

The unified view on secretion is extended to phagocytosis by the use of clostridial toxins. This process is triggered by close apposition of the leukocytes membrane onto an invading microorganism. Phagocytosis was shown to be associated with increase in cell surface area by exocytosis. Selective cleavage of components of the secretory machinery by microinjection or transfection of bacterial neurotoxins (see 4.2) induced an inhibition of phagocytosis (Hackam *et al.*, 1998). These observations indicate that SNARE proteins participate not only in trafficking and exocytosis but also in particle internalization during phagocytosis.

6. CLOSTRIDIAL TOXINS AS THERAPEUTIC TOOLS

6.1. A Clinical Perspective

A large survey on treating patients with BoNT/A showed that there is a long term improvement in patient for about 3–4 months after a single injection. The most commonly used treatments are for patients suffering from craniocervical muscle spasms. The toxin produces chemical denervation of the muscle, thereby causing atrophy and weakness. Studies have shown that even a single injection is an effective therapy for focal dystonias, particularly blepharospasm, hemifacial spasm, and torticollis. It is possible that treatment with BoNT/A will be extended to other disorders in which focal weakening of selected muscles could be useful (Tim and Massey, 1992). To evaluate distant effects of BoNT, single fibre electromyography was performed in patients injected with BoNT/A for hemifacial spasm. An increase of fibre density was recorded at about six weeks after the treatment. The data confirm distant effects on neuromuscular transmission and on autonomic function (Girlanda *et al.*, 1992). The efficiency of the treatment was evaluated in a study of over 250 patients with focal dystonia and hemifacial spasm. The functional improvement is about 80% with very mild side effects. Examination of muscle from patients with blepharospasm who received BoNT/A repeated injections revealed characteristic “sprouting” of the motor axons (Holds *et al.*, 1990). This study ensures that even before a complete understanding of the effect of BoNT/A on the motor system is available, local BoNT/A injections are a preferable choice in symptomatic treatment of focal dystonia and hemifacial spasm (Berardelli *et al.*, 1993).

6.2. In Model Systems

In intact neurons, BoNT A cleaves SNAP-25, and BoNT/C1 cleaves both syntaxin and SNAP-25. In developing mouse spinal cord neurons in culture BoNT/C1 was found to be neurotoxic. In cultures, after BoNT/C1 exposure, synaptic terminals become enlarged and subsequently the cell bodies degenerate. Electron microscopy confirms that early degenerative changes occur in synaptic terminals. This effect is specific to BoNT/C1 and in the same culture BoNT/A has no toxic effect on neurite outgrowth or on neuron survival. This neurodegeneration induced by BoNT/C1 may be used in clinical treatment of dystonia. The mechanism underlies such neurotoxic effect is not known (Williamson and Neale, 1998).

The effect of BoNT/A was compared on both extra- and intrafusal muscle fibers of rats. Four days after injection no action potentials were

elicited with stimulation single-fiber electromyography on the injected side. However, two weeks later, synaptic activity became measurable and was increased on the injected side. Atrophy was evident in all tested muscle fibers 14 days postinjection. This was shown by an increase in cholinergic terminal innervation, end-plate spreading of cholinesterase, and increasing density of small fibers (Rosales *et al.*, 1996). It is expected that advanced optic technologies and the developing of vital dyes will be used to follow structural and functional changes in animal models treated with Clostridial toxins.

7. FUTURE PERSPECTIVE

We discussed two separate groups of toxins which affect secretion— α -Latrotoxin and Clostridial toxins. The mode of action of α -latrotoxin is still not known. However, it is expected that the recent discovery of its receptors will help in resolving this problem. The situation with Clostridial toxins is nearly opposite. Currently, we have a good view of the mode of action of clostridial toxins. However, their receptors and the cell biological process that are involved in the intoxication are not yet known.

The extensive use of Clostridial toxins ranges from basic research to a daily use in the clinic. The effect of clostridial toxins on exocytosis is largely explained by their activity on the SNAREs as discussed throughout this chapter. Indeed, these toxins were used in various experimental setting as molecular probes for secretion. Structural information on SNARE and on their associated proteins should be evaluated in view of the biophysics and kinetic properties of fusion. The Clostridial toxins are expected to be instrumental in such studies. In addition, detailed knowledge of the toxin-target interaction should also lead to progress in sophisticated therapeutic applications of these toxins.

ACKNOWLEDGMENTS. I thank Y. Bladi for helpful discussion. Special thanks to R.M. Toder and L. Naronait for their endless support. This work was supported by the Israel Ministry of Health and the National Institute of Psychobiology.

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Chapter 3

Annexins and Membrane Fusion

Helmut Kubista, Sandra Sacre, and Stephen E. Moss

1. INTRODUCTION

The discovery of the annexins resulted from various, often untargeted experimental approaches involving a large number of different cellular systems. Prior to the introduction of the annexin nomenclature by Crumpton and Dedman (1990), these proteins were therefore known by names that reflected their various biochemical attributes and supposed functions. The first annexin described in the literature was synexin (annexin VII) in 1978, by investigators interested in the role of Ca^{2+} in secretion. Creutz and co-workers (Creutz *et al.*, 1983; Creutz, 1981; Creutz *et al.*, 1978) performed a search for proteins involved in exocytosis in the adrenal medulla, and besides synexin, identified several other annexins (annexins I, II, IV, VI) in Ca^{2+} -dependent binding assays of adrenal medullary cytosol on immobilized granule membranes, and called them chromobindins. Interestingly, proteins with similar properties were also isolated from various tissue homogenates on columns of phenothiazine-Sepharose and were at that time called calcimedins. Since no direct interaction with phenothiazines was observed, isolation of these proteins was explained by the binding to residual membrane vesicles in the cytosol preparations (Geisow and Walker, 1986).

HELMUT KUBISTA, SANDRA SACRE, and STEPHEN E. MOSS Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

Antisera raised against a Ca^{2+} -dependent membrane binding protein named calelectrin from the electric organ of the ray, *Torpedo marmorata*, also recognized chromobindins and calcimedins, as well as proteins that were found to bind to membrane cytoskeletons in a Ca^{2+} -dependent manner (Burgoyne and Geisow, 1989). These immunological relationships hinted at the existence of a family of evolutionarily conserved Ca^{2+} -binding proteins, a notion confirmed in 1986, when cloning and sequencing studies revealed that these independently identified proteins comprised a new protein family distinct from the EF-hand family of Ca^{2+} -binding proteins exemplified by calmodulin (Weber and Johnsson, 1986). Moreover, the abundance of annexins in active secretory tissues, their Ca^{2+} -dependent binding to cytoskeletal elements as well as to plasma- and vesicular-membranes, together with their ability to potentiate vesicle aggregation (see below), raised the possibility that these proteins might have roles in vesicular traffic and ultimately membrane fusion events in exocytosis and endocytosis. Since then the proposed implication of annexins in processes involving membrane fusion has been underlined by many studies. However, the pivotal question in recent times has become whether the functions of annexins herein remain restricted to vesicle aggregation and/or linkage of vesicles to the cytoskeleton and plasma membrane, or whether annexins are involved in the actual fusion process itself. In this chapter we first discuss the biochemical and structural evidence that annexins bind to, aggregate and fuse natural and artificial membranes (see section 2.1). In sections 2.2 and 2.3 we discuss experimental evidence for various roles annexins may play in membrane fusion processes and extend this discussion to consider the ways in which annexin function may be regulated by phosphorylation and nucleotides (2.2 and 2.3.1). In section 3 we review increasingly convincing evidence that annexins function in the endocytic pathway. In section 4 we consider the special case of phagocytosis in neutrophils, and in section 5 we discuss the potential roles of annexins in exocytosis and the role of annexin XIIIb in vectorial vesicle trafficking in polarized epithelia. Finally, we examine data that argue against roles for annexins in vesicle trafficking (section 6), and consider to what extent these proteins meet the requirements needed to act as essential factors in membrane fusion (sections 7 and 8).

2. ANNEXINS IN MEMBRANE FUSION

2.1. The Structural Basis of Annexin-Membrane Interactions

Annexins comprise a distinct family of Ca^{2+} -binding proteins. Each annexin molecule has multiple Ca^{2+} -binding sites which can be classified

into type II and III sites and which are different from helix-loop-helix Ca^{2+} -binding sites (type I) typical of the superfamily of EF-hand Ca^{2+} -binding proteins. The primary structure of all annexins is characterized by four repeats (I–IV) of highly conserved 70 amino acid domains (the only exception is annexin VI which has eight repeats), forming the protein core. Each repeat folds into 5 α -helices (A to E), which are themselves wound into a right-handed superhelix. The helices are connected through short loops or turns. The four repeats are arranged in a slightly curved cyclic array, giving the three dimensional structure of the annexin protein core the shape of a concave disk with a central hydrophobic pore and with the Ca^{2+} -binding sites situated on the convex side (Liemann and Huber, 1997) (Figure 1).

A so-called “calcium bridging” mechanism (Swairjo *et al.*, 1995), where Ca^{2+} is jointly co-ordinated by the annexin Ca^{2+} -binding site and phospholipid head groups, was identified as the mechanism that enables all annexins to bind to negatively-charged phospholipids, such as those enriched in the inner leaflet of the plasma membrane (Liemann and Huber, 1997). Ca^{2+} affinity constants for membrane-binding of annexins therefore always depend on the phospholipid composition of the membrane investigated, and are meaningless without that information (Raynal and Pollard, 1994). The highest Ca^{2+} affinity of annexins is invariably observed in the presence of liposomes or artificial membranes containing acidic phospholipids such as phosphatidylserine (PtdS), phosphatidylethanolamine (PtdE), phosphatidic acid (PtdA) and phosphatidylinositol (PtdIns) which are preferentially localized on the cytoplasmic face of plasma membranes and secretory granule membranes. Membrane binding leads to modest conformational changes which result in the transformation of the slightly curved annexin molecule into a planar disc (Bitto and Cho, 1998), and may also lead to formation of a second phospholipid binding site on the concave side of the molecule, possibly involving the N-terminus (de la Fuente and Ossa, 1997).

In contrast to the highly conserved C-terminal protein core, the N-terminus (or tail) displays high variability among annexins and ranges in length from 11 to 19 (short tail annexins, e.g. III, IV, V, VIII) to more than 100 residues (annexins VII and XI) (Gerke and Moss, 1997). The N-terminal, which is located at the concave side of short tail annexins, is thought to confer functional individuality to annexins. Some annexins appear to have specific protein ligands, e.g. members of the S100 family of EF-hand Ca^{2+} -binding proteins, to which they bind via the N-terminus. In the annexin II₂/p11₂-heterotetramer a dimer of the S100 protein p11 (S100A10) links two annexin II molecules. Note that p11 is unique within the S100 protein family in that it contains no functional Ca^{2+} -binding sites. Its interaction with annexin II is thus Ca^{2+} -independent. This is in contrast to the complexes formed between annexin I-S100C and annexin XI-S100A6

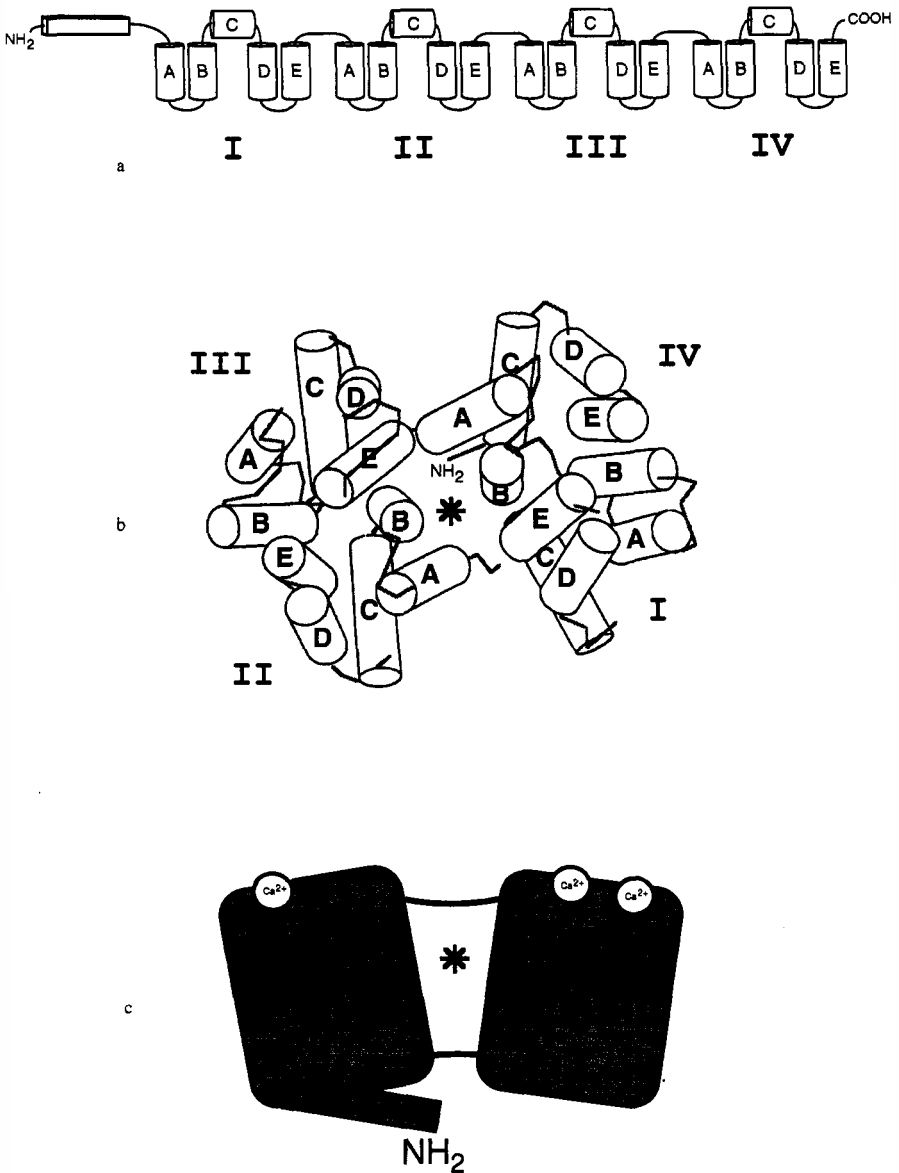


FIGURE 1. Structural organization of an annexin molecule. a) The four C-terminal repeats (I, II, III & IV) each consist of five alpha-helices (A-E). Helices A, B, D and E are oriented (anti-) parallel, whereas helix C lies perpendicular to them. b) Stereoview of an annexin V molecule, viewed from the convex membrane-binding site, showing the central pore (asterisk) (modified from Voges *et al.* (1995)). c) Schematic drawing showing the side-view of an annexin molecule. The Ca²⁺ binding sites are located on the convex side of the molecule, whereas the N-terminus resides on the concave side.

(calyculin) (Gerke and Moss, 1997). Binding of annexin II to p11 is necessary to target annexin II to the submembraneous cytoskeleton but the physiological significance of the other annexin-S100 interactions is less clear.

Other protein ligands include a number of cytoskeletal or cytoskeletally-associated proteins, among them filamentous actin (ligand to annexins I, II and VI), members of the non-erythroid spectrin family (ligands for annexins II and VI) and GFAP (glial fibrillary acidic protein, ligand for annexin II), to which annexins may bind via their C-terminal core rather than their N-termini. Structural analysis of membrane-bound annexins (annexins II, V and VI) by X-ray crystallography and electron microscopy revealed that these molecules behave as peripheral membrane proteins (Lambert *et al.*, 1997; Liemann and Huber, 1997; Burger *et al.*, 1996; Karshikov *et al.*, 1992). These findings question the original suggestion by Pollard and co-workers that annexin VII does not merely adhere to the bilayer but enters it. This earlier view was based on the observation that calcium-activated synexin raises membrane capacitance and displays ion channel activity in patch clamp experiments on phospholipid bilayers (Pollard *et al.*, 1990). Although ion channel activity was also observed for annexins II, V and VI in similar experiments, the conflict with the crystal structure of the membrane-bound molecules presented a major conceptual barrier. One model devised to resolve this conflict was the idea of "micro-electroporation", where the strong electrostatic field displayed by the annexin molecule on the membrane-binding surface would invoke formation of membrane pores, or at least transiently disordered membrane areas, allowing ion flux across the phospholipid bilayer (Demange *et al.*, 1994; Karshikov *et al.*, 1992). The question of whether ion channel activity is due to membrane spanning annexin molecules, monomeric or polymeric (Moss, 1995), or membrane "micro-electroporation" is still unresolved. Recently, the discussion has been rekindled by the finding that annexin XII at low pH undergoes pronounced conformational changes with the formation of single continuous α -helices from helix-loop-helix motifs that appear to insert into the bilayer (Langen *et al.*, 1998b). The proposed model for annexin XII at low pH suggests 7 membrane-spanning α -helices which line a central pore, a structural arrangement that closely resembles the three dimensional organization of other ion channels. It is not known however, whether at neutral pH, ion channel activity of annexins occurs in the membrane-attached configuration or in a membrane-spanning conformation possibly induced by factors other than pH.

The existence of integral membrane forms of annexins is certainly consistent with a number of studies describing Ca^{2+} -independent binding of annexins to membranes (see for example Bianchi *et al.*, 1992). In cell lysates a pool of annexin molecules (e.g. annexins I, II, V, VI and XIIIb) is often found associated with membranes in a detergent-soluble but EGTA-

resistant manner (Lafont *et al.*, 1998; Liu *et al.*, 1997). In BHK (baby hamster kidney) cells, 50% of total annexin II was found associated with membranes in a Ca^{2+} -independent but cholesterol-dependent manner, implicating a role for annexin II in organizing cholesterol-rich membrane domains, e.g. caveolae (Harder *et al.*, 1997). An intact N-terminus (annexin II) or N-terminal modifications, such as phosphorylation (annexin I) and myristoylation (annexin XIIIa and XIIIb) have been shown to be implicated in Ca^{2+} -independent membrane association, which may also be due to binding to other membrane associated proteins as suggested for annexin II. Partial or complete protein insertion into the phospholipid bilayer was suggested for annexin V at conditions of low pH (Kohler *et al.*, 1997), and for annexin I at pH 6.0 (Rosengarth *et al.*, 1998) consistent with studies on annexin XII discussed above (Langen *et al.*, 1998b). There may thus be several mechanisms by which membrane-association of annexins can become independent of Ca^{2+} .

In the absence of Ca^{2+} annexins exist as monomers in solution. However, in the presence of Ca^{2+} annexins tend toward self-association, both in solution and on membranes. In crystals, a variety of quaternary states, such as monomers, dimers (annexin VII), trimers (annexin V) and hexamers (annexin XII) have been described (Langen *et al.*, 1998a). Annexin V trimers were also observed on lipid monolayers using electron microscopy (Voges *et al.*, 1994). Self-association appears to be one means by which annexins could induce granule aggregation. Annexin VII was suggested to bind to granule membranes upon addition of Ca^{2+} and then to self-associate with other membrane bound annexin VII molecules, thereby linking the opposing membranes. Annexin II employs yet another mechanism to link membranes with one another. In a heterotetrameric complex two annexin II molecules are bound at opposing ends to a dimer of the 100 protein p11, creating the potential for the heterotetramer to interact with two membranes simultaneously. Vesicle aggregation can also be accomplished by monomeric annexins. A possible mechanism underlying this phenomenon was recently shown for annexin I. Upon membrane-binding of a single annexin I molecule, conformational changes occur on the concave side of the protein, possibly involving the N-terminus, resulting in the formation of a second phospholipid-binding site which allows the molecule to interact with two different membranes (de la Fuente and Ossa, 1997; de la Fuente and Parra, 1995). The current perception is therefore that the vesicle aggregation capability of annexins is due both to protein-protein interactions of annexin molecules bound to different vesicles and to a bidentate binding mode of single annexin molecules (Bitto and Cho, 1998).

From these data it appears that annexins exist *in vivo* in a variety of different conformations (Figure 2): i) soluble in the cytosol, ii) bound

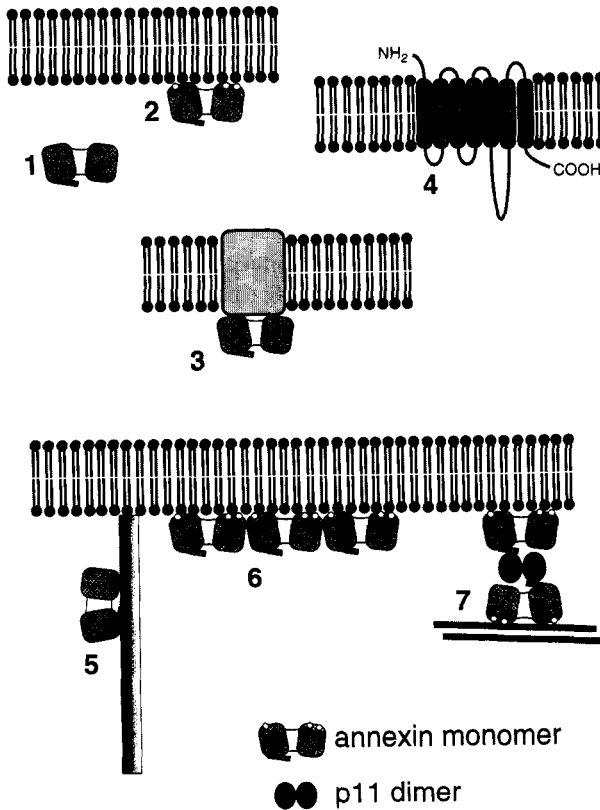


FIGURE 2. Various conformations annexins may acquire *in vivo*. Annexin molecule(s): 1, soluble in the cytosol; 2, bound to phospholipid membranes in a Ca²⁺-dependent manner via the "Ca²⁺ bridging" mechanism; 3, bound to membranes in a Ca²⁺-independent manner, e.g., via a membrane inserted protein ligand; 4, inserted into the membrane in a refolded conformation (this model assumes that domains I, II and IV each form two membrane-spanning helices from helices A/B and D/E, respectively, whereas due to the absence of conserved residues domain III is thought to provide only one membrane-spanning helix formed from helices A and B of the membrane attached protein, but see Langen *et al.* (1998b) for details. Location of N- and C-terminus of the membrane-inserted annexin molecule are unknown. The orientation shown is therefore arbitrary); 5, bound to protein ligand, e.g., cytoskeletal elements; 6, self-associated forming a membrane-bound homopolymer; 7, forming a heteropolymer with the S100 protein p11, which crosslinks membrane and, for example, cytoskeleton (in the case of annexin II).

peripherally to membranes in a Ca^{2+} -dependent manner, iii) bound to membranes in a Ca^{2+} -independent manner, iv) inserted into membranes, v) bound to protein ligands and vi) as homo- or hetero-polymers. Not all of these possibilities are mutually exclusive. Thus, it is possible that an annexin that is associated Ca^{2+} -independently with a membrane, may in fact be inserted into the membrane.

2.2. Modulation of Annexin-Membrane Interactions by Phosphorylation

Given that many signaling events that lead to exocytosis and endocytosis also lead to the activation of protein kinases, it is reasonable to expect that annexins might serve as substrates for protein kinases *in vivo*, and that phosphorylation would modulate some aspects of their membrane-binding or fusogenic properties. Annexins are targets for protein tyrosine kinases (PTK) of both the receptor-PTK and non-receptor-PTK type as well as serine/threonine kinases (reviewed recently by Rothhut (1997)). The EGF receptor kinase and pp60^{c-src}, for example, phosphorylate annexin I on Tyr-21 in the N-terminal domain. The PDGF receptor kinase and pp60^{v-src} phosphorylate annexin II on Tyr-23. Annexins I and II also have phosphorylation sites for conventional protein kinase C (PKC) at Thr-24, Ser-27, Ser-28 and Thr-41 (annexin I) and Ser-25 (annexin II) respectively. PKC phosphorylation has also been demonstrated for annexins VII, VIII and XI, whereas annexins V and VI do not appear to be PKC substrates *in vivo*. In fact, annexins V and VI have been shown to act as PKC inhibitors.

With the exception of a protein kinase A phosphorylation site at Thr-216 in annexin I, all phosphorylation sites identified to date are situated in the N-terminal domains of annexins (Rothhut, 1997). Phosphorylation of annexins has been shown to alter their lipid-binding characteristics, membrane aggregating properties, protein ligand-binding as well as subcellular distribution. For example, binding to chromaffin granules and to p11 are both impaired after phosphorylation of annexin II by PKC (Regnoui *et al.*, 1995; Johnsson *et al.*, 1986). Phosphorylation of annexin II by pp60^{v-src} on the other hand decreases the affinity of binding to phosphatidylserine liposomes, but leaves the interaction with p11 unaffected (Powell and Glenney, 1987). Phosphorylation of annexin I by the EGF receptor kinase causes as much as a 5-fold reduction in the amount of Ca^{2+} required for half-maximal association with phospholipids (Ando *et al.*, 1989), or phosphatidylserine vesicles (Schlaepfer and Haigler, 1987).

In MVBs (multivesicular bodies) annexin I is associated with membranes in a Ca^{2+} -independent manner, but is converted by EGF receptor kinase-phosphorylation into a form that requires Ca^{2+} for membrane association (Futter *et al.*, 1993). Phosphorylation also affects the membrane

aggregation properties of annexins. PKC-phosphorylation of annexins I (Wang and Creutz, 1992) and annexin II (Johnstone *et al.*, 1992), for example, inhibits vesicle aggregation, possibly by decreasing the Ca^{2+} -affinity of the process, whereas lipid-binding characteristics were virtually unaltered. However, Regnoui *et al.* (1995) did not observe induction of aggregation by PKC-phosphorylated annexin II at any concentration of Ca^{2+} . Moreover, in their study phosphorylation of annexin II decreased the affinity of binding to chromaffin granules. The importance of the N-terminus in determining Ca^{2+} requirement, phospholipid binding and vesicle aggregation is obvious from studies with N-terminally-truncated annexins. Full-length annexin I requires $30\mu\text{M}$ Ca^{2+} , whereas the N-terminally-truncated protein requires only $5\mu\text{M}$ Ca^{2+} for binding to a phospholipid column (Ando *et al.*, 1989). However, half-maximal Ca^{2+} values for binding to granule membranes were not affected for similar truncated proteins in the study of Wang and Creutz (1994), but these proteins did show altered Ca^{2+} sensitivities in a membrane aggregation assay.

N-terminal alterations (phosphorylation/truncation) may also affect the subcellular localization of annexins. Annexin XI was found localized in the nucleus of both an embryonic fibroblast cell line (3YI cells) and undifferentiated mesenchymal cells of the rat embryo. In adult rat tissues nuclear localization of annexin XI was uncommon (Mamiya *et al.*, 1994). In 3YI cells transformed by the Rous sarcoma virus oncogene (SR-3YI cells) more annexin XI was phosphorylated, the phosphorylated protein had lost its binding ability to phosphatidylserine vesicles and it was now found in the cytosol (Mizutani *et al.*, 1993). Full length annexin XI also localizes predominately to the nucleus of COS-7 cells, but its N-terminally truncated derivative is found in the cytosol (Mizutani *et al.*, 1995). These findings indicate that the N-terminal tails of annexins can function as localization signals, and can be regulated by phosphorylation. Collectively, these studies show that phosphorylation is an important post-translational modification of annexins that is likely to be involved in regulation of their activities.

2.3. Annexins and Membrane Fusion in Exocytosis

Membrane fusion is a crucial part of vesicular trafficking, be it exocytosis, endocytosis, phagocytosis, pinocytosis or transcytosis. Involvement of annexins in these processes has been most extensively studied for exo- and endocytosis. In exocytosis, vesicles are transported from the Golgi apparatus to the plasma membrane via the cytoskeleton. There, vesicle and plasma membranes fuse with each other. In regulated exocytosis in many cell types, secretory vesicles dock to the plasmalemma via v-SNARE/t-SNARE-interactions. Vesicles are then primed to the exocytotic machinery via Mg-

ATP dependent reaction(s). Docked and primed secretory vesicles are inhibited from spontaneous fusion by a molecular “fusion” clamp, which is only released by a rise in cytosolic free Ca^{2+} , usually evoked by secretagogues. This arrangement allows secretion to occur almost instantaneously once cellular Ca^{2+} starts to rise and is of crucial importance in synaptic exocytosis where rapid release of neurotransmitter is essential.

In other cell types, exocytotic secretion is not only regulated by Ca^{2+} , but also by guanosine triphosphate (GTP) and protein kinase C activation (Pollard *et al.*, 1998) arguing for the involvement of either a membrane fusion protein that is activated by both Ca^{2+} , GTP and protein kinase C, or multiple proteins, each of which acts as a sensor for one of these regulatory elements and whose activities are co-ordinated during secretion. Some cell types, e.g. chromaffin cells, possess a cortical actin cytoskeleton, which prevents secretory vesicles from direct interaction with the plasma membrane. Therefore, dissolution of the cortical actin web is required for secretion to ensue. In compound exocytosis, an intact secretory granule makes contact and fusion with the membrane residue of a granule which has just completed a fusion event (Pollard *et al.*, 1992). Compound exocytosis is distinguished from unitary granule exocytosis, as seen for example in neurotransmitter release and tends to occur more slowly.

Annexins were first suggested by Zaks and Creutz (1990a) to meet some important prerequisites to play a role in exocytosis. They are expressed in all exocytotic tissues, they interact with both vesicular and plasma membranes and they are Ca^{2+} -dependent molecules. From the above considerations, taken together with what we know about exocytosis, there are multiple sites where annexins may have roles. In sections 2.3.1 to 2.3.7 we consider the experimental evidence that supports roles for annexins in exocytosis.

2.3.1. A Membrane Fusion Protein Activated by Ca^{2+} , GTP and Protein Kinase C

Annexins are Ca^{2+} -binding proteins and as such they have a key attribute to function as Ca^{2+} -responsive elements linking the rise of cytosolic free Ca^{2+} to membrane fusion in regulated exocytosis. Some annexins (like annexins V and VII) require Ca^{2+} in the high micromolar range for half-maximal binding to phospholipids. However, of all known annexin family members annexin II, in its heterotetrameric configuration, has the lowest Ca^{2+} requirement (in the submicromolar range) for binding to phospholipid vesicles. The Ca^{2+} concentration at which annexins bind Ca^{2+} or achieve half-maximal membrane binding covers a wide range and depends on the phospholipid composition. The process of exocytosis probably

involves distinct sequential steps with different Ca^{2+} requirements between 0.1 μM and 1 mM (Zaks and Creutz, 1990a). Pollard *et al.* (1998) postulated a Ca^{2+} site of action in exocytosis from chromaffin cells with a K_D in the 50 to 200 μM range. Ca^{2+} -dependent neutrophil secretion apparently involves two distinct mechanisms that differ in their affinity for Ca^{2+} (Rosales and Ernst, 1997). We can conclude therefore that both low and high affinity annexins may come into play at some exocytotic step.

Besides Ca^{2+} , annexins have also been reported to interact with nucleotides. Annexins I, IV and VI bind ATP, and annexins VI and VII bind GTP (Bandorowicz Pikula, 1998; Han *et al.*, 1998; Bandorowicz Pikula and Awasthi, 1997; Bandorowicz Pikula *et al.*, 1997). Moreover, annexin VII has been reported to hydrolyze GTP in a Ca^{2+} -dependent manner and in a sweeping functional *coup d'etat*, was proposed to be an atypical G-protein responsible for both detecting and mediating the Ca^{2+} /GTP signal for exocytotic membrane fusion (Pollard *et al.*, 1998). Nucleotide phosphodiesterase activity has been most extensively investigated for plant annexins (Delmer and Potikha, 1997; Calvert *et al.*, 1996) although the turnover rate is much lower than that which is normally associated with a *bona fide* enzyme. Nevertheless, there is no doubt that nucleotide-binding by annexins occurs *in vitro*, although whether this extends to living cells is not known. Undoubtedly, defining the nucleotide-binding site in an annexin would aid progress in this area, opening the way for rational mutagenic strategies to investigate this phenomenon in whole cells.

As discussed in section 2.2, annexins I and II are good substrates for PKC (Rothhut, 1997) and several studies have shown phosphorylation of these annexins by PKC modulates vesicle binding/aggregation. Moreover, in permeabilized chromaffin cells, prior phosphorylation of annexin II by PKC was required for annexin II to maximally counteract secretory rundown (Sarafian *et al.*, 1991). From these data, it appears that although no single annexin is likely to act as a Ca^{2+} -, GTP- and protein kinase C-regulated membrane fusion protein, a combination of annexins might provide all the elements necessary to function in exocytosis *in vivo*.

2.3.2. Docking of Secretory Granules to the Plasma Membrane by Annexins

In anterior pituitary cells, few secretory granules are located beneath the plasma membrane in the region where the peripheral cytoplasm is occupied by numerous subcortical actin filaments, whereas in the region free of the subcortical actin filaments many secretory granules lie in contact with the plasma membrane and are linked by intervening strands. Approach of the secretory granules to the plasma membrane appears to be controlled

by the subcortical actin filaments (Senda *et al.*, 1994; Aunis and Bader, 1988). In vitro, annexin II₂/p11₂ catalyses calcium-dependent bundling of F-actin possibly involving the region between residues 286–294 of annexin II which is strongly homologous to the actin-binding domain of myosin (Donnelly and Moss, 1997; Jones *et al.*, 1992). By interacting with F-actin, annexin I may therefore clear a path for secretory vesicles to move to the plasma membrane, allowing fusion to occur (Donnelly and Moss, 1997). In these cells, annexin II was found associated with the plasma membrane and with membranes of the secretory granules (Senda *et al.*, 1998). Annexin II was also found localized at the granule to granule contact sites, suggesting that it is one of the components of the 7 to 8 nm intergranular bridges. Formation of these bridges may be an essential tethering step, bringing granule membranes sufficiently close to one another to enable fusion to subsequently occur. They are absent at the fusion sites however, and may therefore not be involved in the fusion process itself.

2.3.3. Annexins and Vesicle Aggregation

The ability of an annexin to aggregate secretory granules was first shown for annexin VII by Creutz and co-workers (Creutz *et al.*, 1978), and has been confirmed for most of the other annexin family members (Creutz 1992). Annexin V on the other hand, at least at neutral pH, is aggregation-inactive (Hoekstra *et al.*, 1993), whereas annexin VI inhibits granule aggregation (Zaks and Creutz, 1990b). It was suggested that Ca²⁺ causes annexin VII to undergo significant conformational changes leading to the exposure of hydrophobic residues, that could in turn lead to polymerization. In the model for annexin VII-mediated vesicle aggregation, two annexin VII molecules initially form a dimer, and this then undergoes further polymerization with simultaneous engagement of two adjacent membranes (Pollard *et al.*, 1992).

The N-terminus seems to play an important role for the aggregating activity of annexins, although because the N-terminus of annexins lies on the opposite side of the molecule to the phospholipid-binding sites, the structural basis of this activity is not readily explained. This was shown for annexin I. Preincubation of annexin I with a monoclonal antibody directed against the N-terminal region did not prevent binding to vesicles but abolished its vesicle aggregation capability (Meers *et al.*, 1992). Both PKC phosphorylation and cleavage of the N-terminus of annexin I also inhibited aggregating activity but do not affect Ca²⁺-dependent membrane-binding (Wang and Creutz, 1994; Wang and Creutz, 1992). In addition, a chimaeric protein consisting of the N-terminus of annexin I and the C-terminal core of annexin V has aggregating activity, whereas native annexin V is inactive in aggregation assays (de la Fuente and Parra, 1995).

Annexin self-association or formation of a heterotetramer (annexin 11) were suggested to be required for membrane contact at low Ca^{2+} levels, whereas at higher Ca^{2+} levels a single annexin molecule may ligate the two membranes (Zaks and Creutz, 1991). For example, although both annexin II monomer and heterotetrameric annexin $\text{II}_2/\text{p11}_2$ are able to mediate the aggregation of liposomes composed of phosphatidylserine, annexin $\text{II}_2/\text{p11}_2$ requires 20-fold less Ca^{2+} . At 1mM Ca^{2+} aggregation of lamellar bodies mediated by annexin $\text{II}_2/\text{p11}_2$ was four-fold greater than that induced by monomeric annexin II (Liu *et al.*, 1995). In keeping with these findings, at low micromolar Ca^{2+} concentrations heterotetrameric annexin $\text{II}_2/\text{p11}_2$ aggregates chromaffin granules, whereas monomeric annexin II lacks this ability (Drust and Creutz, 1988).

Cryo-electron microscopy of junctions formed by annexins between lipid membranes indicates that both heteropolymeric annexin complexes, monomeric annexins and annexin homopolymers may be involved in membrane aggregation. In the case of junctions formed by the annexin $\text{II}_2/\text{p11}_2$ -heterotetramer, each annexin II molecule binds to the outer lipid leaflet of one membrane by its convex side and to the p11 dimer by its N-terminus at the concave side. Other junctions may be formed when monomeric annexins I and II simultaneously bind to two membranes by both their convex and concave sides, or alternatively, when annexin molecules bound with their convex side to only one of the two membranes interact laterally with annexins attached to the juxtaposed membrane. Yet another type of junction observed appears to be due to two annexins bound with their convex sides to the lipid membrane which interact with one another via their concave faces (Lambert *et al.*, 1997) (Figure 3).

2.3.4. Annexins and the Organization of Membrane Microdomains

In many cells, regulated exocytosis requires a pool of PtdInsP_2 at the release sites (Zheng and Bobich, 1998). Cholesterol-rich membrane domains may provide a means of compartmentalizing not only such signaling lipids (Pike and Miller, 1998) but perhaps also secretory proteins. Given the preferences exhibited by annexins for certain phospholipids over others, it is possible that a two-dimensional lattice of annexin molecules, perhaps in the triskelion arrangement described earlier (section 2.1), could generate a membrane microdomain enriched in one particular phospholipid (Figure 4). This idea is supported by data showing that binding of annexin IV to mixed lipid membranes induces segregation of phosphatidylglycerol and phosphatidylcholine (Junker and Creutz, 1993). Moreover, a pool of Ca^{2+} -independently bound annexin II localizes to cholesterol-rich domains and is released together with actin and actin-binding proteins upon administration of cholesterol-clustering agents

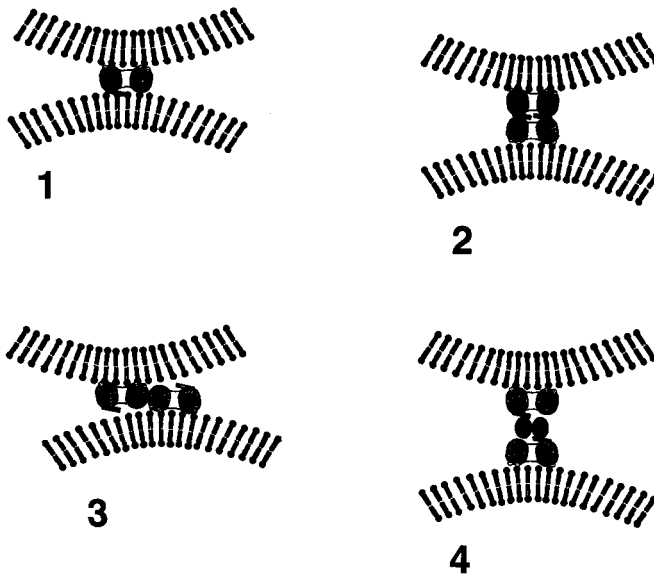


FIGURE 3. Annexins aggregate membranes by formation of various junctions. 1, a single annexin molecule binds to phospholipids by both its convex and concave side, thereby interacting with two juxtaposed membranes; 2, junction formed by two annexin molecules that are bound to membranes via their convex sides and interact with each other via their N-terminal bearing concave side; 3, junction formed by lateral interactions of single annexin molecules that interact with membranes only by their convex sides; 4, junction formed by the annexin II₂/p11₂-heterotetramer, where annexin II molecules bind to membranes via their convex faces and are linked on their concave sides by a p11-dimer.

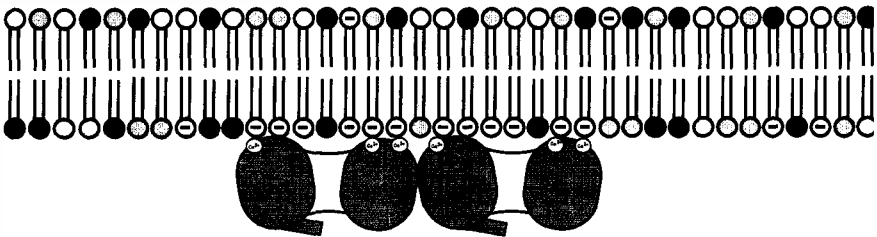


FIGURE 4. Lipid segregation by annexins. Annexins preferentially bind via Ca^{2+} ions to negatively charged phospholipids. Binding of annexins to the membrane is therefore thought to induce a clustering of negatively charged phospholipids in the plane of the cytosolic leaflet which elsewhere retains a mixed lipid composition. The segregation-effect is augmented by self association of membrane-bound annexins, generating microdomains of specific lipid composition.

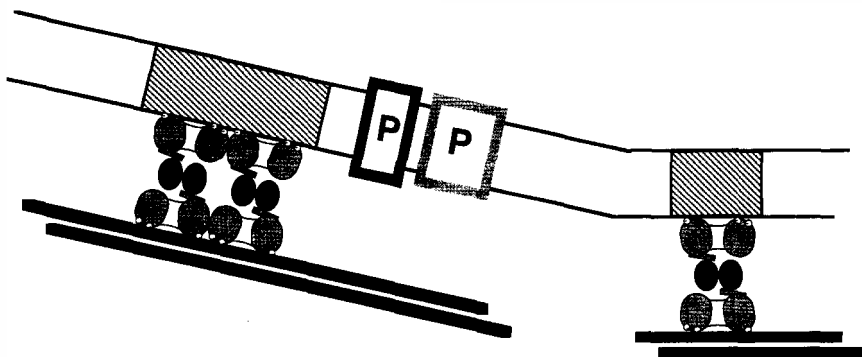


FIGURE 5. Putative role of annexins in the organization of membrane microdomains. The annexin II/p11₂-heterotetramer crosslinks membrane areas of specific phospholipid composition (cross-hatched areas) to the cytoskeleton, thereby restricting the diffusion of enclosed phospholipids and proteins (P).

(Harder *et al.*, 1997). Annexin II, possibly in its heterotetrameric form, is therefore thought to participate in linkage of cholesterol-rich membrane domains to the actin cytoskeleton, causing restricted diffusion and sequestration of phospholipids (Gerke and Moss, 1997) (Figure 5). The question remains as to whether or not annexin II functions to generate the cholesterol-rich domain, or simply becomes recruited to these regions by virtue of binding to peptide components of the protein complex.

2.3.5. Annexins as Membrane Fusogens

A deformable membrane is not favourable for fusion to occur. Apposed membranes must be physically forced together with sufficient energy to bring about membrane reorganization. At the same time, both membranes must be prevented from moving away from one another (Zaks and Creutz, 1990a). In chromaffin granules, osmotically-induced vesicle swelling occurs due to the hyperosmotic core contents and this facilitates fusion. Moreover, for membrane fusion to take place, some perturbation of the membrane phospholipids is required, and this could be provided by an increase in membrane tension or by a “disordering” of the bilayer. Annexins are known to be able to both stabilize and destabilize membranes, depending on the experimental conditions (Gerke and Moss, 1997). It is therefore possible that annexins have a passive role in membrane fusion, simply by adding rigidity to vesicular and/or plasma membranes. Alternatively, if binding of annexins to vesicles or membranes causes

increased perturbation of phospholipids, then this implies a more active role.

The annexin II₂/p11₂ complex was suggested to be involved in stabilizing certain membrane domains through complexation with the underlying cytoskeleton, thus providing rigidity to the plasma membrane (Gerke and Moss, 1997). Moreover, membrane-bound human annexin V shifts the bilayer fluidity gradient in its vicinity to a more immobilized profile, resulting in membrane rigidification of the hydrophobic part of the bilayer close to the phospholipid polar head (Megli *et al.*, 1998). In the presence of Ca²⁺ annexins tend to self-associate rapidly on phospholipid surfaces. Annexins V and VI self-associate into trimers that then form higher order aggregates, resulting in the formation of a protein lattice underneath the plasma membrane. This "annexin lattice" was suggested to play a role in phospholipid sequestration, regulation of membrane fluidity and modulation of the activity of other membrane associated proteins, for example ion channels (see below) (Naciff *et al.*, 1996). It is also easy to imagine that formation of an "annexin lattice" may lead to increased membrane stability.

Fusion in annexin-induced granule aggregates occurs at a very slow rate, but is markedly increased by the addition of *cis*-unsaturated fatty acids (Gerke, 1996). During stimulated exocytosis in many cells, phospholipase A₂ becomes activated, leading to the generation of *cis*-unsaturated fatty acids such as arachidonic acid. Liberation of free fatty acids is thought to increase membrane fluidity thereby increasing the possibility of lipid interactions between apposed membranes. The fact that some annexins can bind arachidonic acid in the presence of Ca²⁺ (Edwards and Crumpton, 1991) raises the possibility that annexins, already located within aggregates of vesicles or plasma-membranes, might sequester the fatty acid into the fusogenic compartment. Annexin V at low Ca²⁺ concentrations was reported to perturb membranes, thus causing loss of vesicle integrity. However, at higher Ca²⁺ concentrations, annexin V exerts the opposite effect and promotes membrane integrity and stability (Goossens *et al.*, 1995). In the case of annexin V it is difficult to reconcile the observation that high Ca²⁺ concentrations lead to a more stable complex between annexin and membrane, with ion channel activity, given that "micro-electroporation" (Figure 6), the mechanism whereby annexin V could function as an ion channel despite being bound only peripherally to the membrane, argues for a membrane destabilizing effect (Liemann and Huber, 1997).

2.3.6. Annexin-Mediated Ion Fluxes in Exocytosis

It is clear that in some secretory cells, Ca²⁺ fluxes from the interior of secretory granules may have a role in fusing granules both to each other

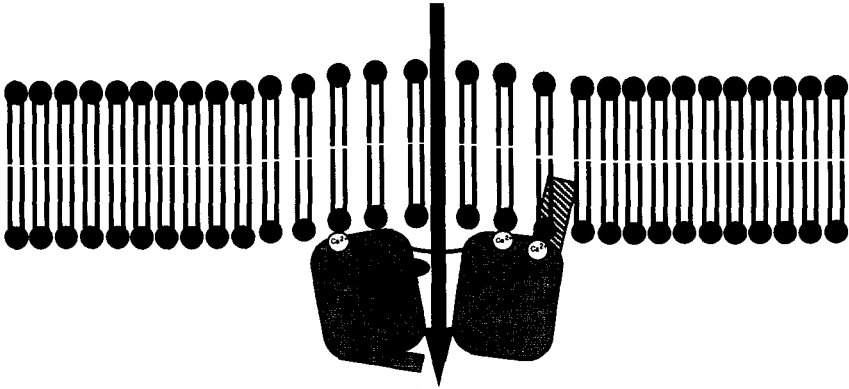


FIGURE 6. Annexin membrane “micro-electroporation”. Monomeric annexin is bound Ca^{2+} -dependently to the inner leaflet of the plasma membrane. The phospholipid bilayer beneath the annexin molecule is disturbed by a strong electric field exerted by the annexin molecule, by the membrane penetrating Trp187 (cross-hatched rectangle) and the interactions between the Ca^{2+} binding loops and the phospholipid headgroups. Due to this disturbance in this localized region the bilayer becomes permeable to ions. Ion selectivity is provided by a filter (SF) located in the central pore, allowing, for example in the case of annexin V, only Ca^{2+} to pass the bilayer.

and also to the plasma membrane. Thus, Ca^{2+} is released from the vacuolar lumen and triggers homotypic vacuole fusion in yeast (Peters and Mayer, 1998). Although a direct involvement of Ca^{2+} in exocytotic membrane fusion is debatable (White, 1992), release of Ca^{2+} from secretory granules may contribute to the cytosolic signal that triggers membrane fusion. *In vitro* several annexins, among them annexins V, VI and VII have been demonstrated to have Ca^{2+} selective ion channel activity (Demange *et al.*, 1994). Moreover, annexin IV (Chan *et al.*, 1994) and annexin VI (Naciff *et al.*, 1996) have been shown to inhibit ion conductances in certain cell types. Clearly, annexins may therefore have roles in the generation and regulation of Ca^{2+} fluxes. Both annexin V and annexin VI, but not annexin I or annexin II (tetramer), release Ca^{2+} from chromaffin granules. Release of Ca^{2+} is not due to a disruption of granule membrane integrity, because there is no concomitant release of catecholamines (Jones *et al.*, 1994). Annexin VI is located at the inner face of the plasma membrane during the exocytotic process, a location also occupied by secretory granules subsequent to dissolution of the actin barrier. It was suggested that a complex between secretory granules, plasma membrane and annexin VI may form, in which annexin VI triggers the release of granular Ca^{2+} , thereby augmenting the

transmembrane Ca^{2+} signal and assisting in the induction of membrane fusion.

2.3.7. Annexin Binding to Secretory Regulators

In addition to the well-characterized S100 family members that serve as intracellular ligands for the annexins, various other less well defined interactions have been described linking annexins with intracellular signaling molecules known to participate in exocytosis. Thus, it is possible that annexins influence exocytosis via interaction with these signaling proteins. For example, annexin I was identified as a putative receptor for activated C kinase (RACK), by binding to PKC in the presence of phosphatidylserine and Ca^{2+} (Mochly Rosen *et al.*, 1991a). A potential PKC-binding on annexin I was shown to comprise the C-terminal 15 amino acids (Mochly Rosen *et al.*, 1991b). Interestingly, synaptotagmin, which has a well-established role in synaptic exocytosis, also interacts with a synthetic RACK peptide that consists of the 15 C-terminal amino acids of annexin I.

The annexin II C-terminus is closely related to that of annexin I and also has similarity with a domain located within 14-3-3 proteins (Roth *et al.*, 1993). This shared domain was suggested to be involved in specific protein-protein interactions. Thus, a 16 amino acid synthetic peptide corresponding to the C-terminus of annexin II prevented 14-3-3 induced stimulation of secretion and partially inhibited regulated exocytosis. Exol is another protein that stimulates secretion in run-down assays of permeabilized chromaffin cells (Burgoyne *et al.*, 1993). Interestingly, Exol was discovered to belong to the family of 14-3-3 proteins. These findings argue for a common target of both annexin II and 14-3-3 proteins in exocytosis and a regulatory role exerted on other proteins by interaction with the shared key domain.

3. ANNEXINS AND MEMBRANE FUSION IN ENDOCYTOSIS

Endocytosis is the process of cellular uptake of substances such as nutrients and growth factors from the external environment, as well as infectious agents targeted for destruction (phagocytosis). Endocytosis requires cells to constantly remodel the plasma membrane in response to external stimuli. The process of endocytotic internalization can be separated into five main sub types; the clathrin-dependent pathway, the caveolar pathway, a clathrin- and caveolin-independent pathway, phagocytosis and macropinocytosis. It seems that the only element common to all of these pathways is involvement of the submembraneous cytoskeleton (Riezman *et*

al., 1997). Even within the clathrin-dependent pathway there are functionally distinct coated pits that develop into coated vesicles. Thus, the temperature dependence for fission of clathrin-coated pits from the plasma membrane differs depending on whether or not the endocytic vesicles contain β_2 adrenergic receptors or transferrin receptors (Cao *et al.*, 1998). Annexins have mainly been investigated in the clathrin-dependent and -independent pathways, and also phagocytosis, so these endocytic routes will be the focus of discussion here. A few studies have also examined annexins in the caveolar-dependent pathway. Caveolae are clathrin-free invaginations that form endosomes, and which contain proteins that have been identified as probably being involved in fusion of vesicles. Annexins II and VI have been identified on caveolae (Schnitzer *et al.*, 1995). Another form of vesicular transport in which annexin XIIIb is implicated is apical transport in certain polarized epithelial cells (Fiedler *et al.*, 1995). This aspect of annexin biology is discussed in section 5.1.6.

Receptor mediated endocytosis is initiated by the binding of a ligand to its cognate cell surface receptor. Receptors may either already be clustered or form clusters following engagement of ligand, these clusters quickly become internalized into invaginated clathrin-coated pits. Membrane invagination occurs through rearrangement of the clathrin lattice, which folds into the cell to form a pit that remains attached to the membrane by a thin membrane stalk. The stalk is then broken, a process in which the GTPase dynamin is implicated (Baba *et al.*, 1995; Damke *et al.*, 1995), or by remodelling of spectrin. Once internalized, these coated vesicles can fuse with one another (Salzman and Maxfield, 1988; Salzman and Maxfield, 1989). Such vesicles lose their coats as they become endosomes, the stage at which segregation of ligands and receptors occurs (Hanover *et al.*, 1984), as well as the sorting of metabolites and other cargo. It is also at this point, when early endosomes are gathering to form the multivesicular body, that the interior of the vesicle becomes acidified by the activity of an ATP-dependent proton pump (Yamashiro *et al.*, 1984) and it is this change in pH that causes ligands to dissociate from their receptors. The method by which an endosome is created can vary but once formed most internalized molecules converge on early endosomes. After sorting in these endosomes, a pool of receptors may be recycled to the plasma membrane while ligands are transferred to lysosomes for degradation. There is some evidence from experiments on the low density lipoprotein (LDL) receptor, that a region of the receptor directs itself for recycling and the ligand for degradation (Chen *et al.*, 1990). Molecules destined for degradation then become concentrated in the late endosomal compartment from where they pass to the lysosomes (Geuze *et al.*, 1983). In some cell types, such as rat basophilic leukaemia cells, lysosomes are functionally indistinguishable from secretory

granules, thus providing a common element of both the exocytotic and endocytic pathways.

Progression along the endocytic pathway demands that fusion events must occur in an ordered fashion between compartments. Membrane fusion is thought to be preceded by the formation of a pore or a collar of protein between the two membranes that are due to fuse together (Monck and Fernandez, 1992). Annexins may have roles at various steps of the endocytic pathway, from budding of clathrin-coated pits to fusion or motility of early and intermediate endosomes and sorting to the lysosomes. These possible roles are discussed below.

3.1. Annexin VI

Studies on the intracellular distribution of annexin VI in hepatocytes revealed this protein to be a major marker of the endosomal compartment. Annexin VI binds to the cytoplasmic face of endosomes in a Ca^{2+} -dependent manner (Jackle *et al.*, 1994) and is enriched on endosomes within the apical part of the cell (Ortega *et al.*, 1998). Similar levels of both isoforms of annexin VI were observed on early, late and recycling endosomes (Jackle *et al.*, 1994). Note that alternative splicing gives rise to two forms of annexin VI, the smaller of which lacks a 6 amino-acid insert close to the start of the seventh repeat (Moss and Crumpton, 1990). In co-localization experiments 50% of endosomes co-containing transferrin also stained positively for annexin VI. These results led to the suggestion that annexin VI may have a role in the recycling and transport of this receptor to late endosomes or lysosomes (Ortega *et al.*, 1998).

Another key step in which annexin VI has been suggested to function is budding of clathrin-coated pits from the plasma-membrane. This is one of the earliest steps in endocytosis, occurring immediately after receptors become gathered in pits (Heuser and Anderson, 1989) coated with a planar lattice of clathrin (Larkin *et al.*, 1986). The aggregated receptors cause an invagination of the lattice until the plasma membrane forms a membraneous stalk (Heuser and Anderson, 1989; van Deurs *et al.*, 1989). The nascent vesicle then stays attached to the membrane in this way until membrane fusion occurs and it becomes sealed as an independent organelle. There have been suggestions that type II secreted phospholipase A_2 (sPLA $_2$) and annexin VI may be involved in the severing of the membrane stalk in the final stages of vesicle formation. Aspects of this process can be reconstituted *in vitro* using isolated plasma membranes. Under these conditions vesicles are able to form up to the point where they are held by a membrane stalk, severing of the stalk then requires the addition of cytosolic factors. It is at this point that annexin VI has been suggested to play a role,

by breaking the stalk when the membranes fuse (Lin *et al.*, 1992). To do this annexin VI and type II sPLA₂ could provide the destabilizing factor and free polyunsaturated fatty acids respectively, that will stimulate both membrane fusion and stalk fission (Koumanov *et al.*, 1997). It is reasonable, at least at a hypothetical level, to postulate that if annexin VI acts as a destabilizing factor at the membrane then this may increase the chance of lipids in the stalk becoming susceptible to hydrolysis. It is already known that binding of annexins to membranes can cause disorder on both sides of the membrane (Huber *et al.*, 1992), as described earlier. Type II sPLA₂ released from secretory granules could act by producing non-esterified fatty acids that are required for increased annexin binding and which aid the fusion process (Burgoyne and Geisow, 1989). The membrane destabilization process could be either enzymatic or structural. In the latter case, annexin VI could interact directly or indirectly with the lipids in the membrane to disrupt the stalk or it could draw together the stalk membranes until they fuse. No other annexins have been demonstrated to have an effect on budding (Lin *et al.*, 1992), and the fact that dynamin appears capable of performing this molecular task raises questions about how or why annexin VI might do likewise.

The studies of Lin *et al.* (1991) implied that annexin VI is required for budding to occur. The steps involved in converting a flat coated membrane into an invagination held to the membrane by a stalk cannot take place without the participation of ATP, one or more cytosolic factors and Ca²⁺. They suggested that Ca²⁺ is required for membrane fusion while ATP may be required for putative phosphorylation reactions. The same group then showed (Lin *et al.*, 1992), using purified plasma membranes, that annexin VI was the cytosolic factor that mediated budding of clathrin-coated pits. Thus, when annexins were depleted from the cytosol almost all budding was eliminated. Re-addition of purified annexin VI to this depleted cytosol almost fully restored budding activity, although the concentration of annexin VI necessary to induce budding was several thousand times greater than is typically found in living cells. Moreover, budding only occurred in the presence of 100 μM Ca²⁺, which is surprising given that in intact cells budding of clathrin coated pits occurs at resting Ca²⁺ levels of less than 1 μM.

In performing these experiments this group were able to show a link between ATP dependence and stimulatory factors in the cytosol (Lin *et al.*, 1992). ATP may be responsible for the activation of annexin VI by phosphorylation, as when it is replaced with ATPγS budding can still occur (Lin *et al.*, 1991), or it could be that ATP-binding by annexin VI (Bandorowicz Pikula *et al.*, 1997; Bandorowicz Pikula and Awasthi, 1997b) modulates some activity of the protein. Indeed, fluorescence spectroscopic studies on

the interaction of annexin VI with nucleotides have suggested that nucleotide-binding by annexin VI can induce a conformational change in the protein (Bandorowicz Pikula and Awasthi, 1997a). Recent work has shown that in the presence of physiological concentrations of ATP, the half-maximal binding of annexin VI to anionic phospholipids occurs at a reduced Ca^{2+} concentration (Tagoe *et al.*, 1994). Binding of ATP to annexin VI has also been shown to increase the Ca^{2+} concentration needed to bind F-actin (Bandorowicz Pikula and Awasthi, 1997a). Clearly, ATP could modulate the function of annexin VI by altering its affinity for Ca^{2+} . If purified annexin VI was added to the depleted cytosol in the absence of ATP then no budding activity was observed (Lin *et al.*, 1992). The role of Ca^{2+} in the budding process is unclear. Under the experimental conditions used annexin VI will undoubtedly bind Ca^{2+} -dependently to the isolated plasma membranes, but this occurs at Ca^{2+} concentrations of $10\mu\text{M}$ or less (Sudhof *et al.*, 1984), and approximately $100\mu\text{M}$ was required for optimal budding activity (Lin *et al.*, 1991).

Conflicting data has emerged from our own studies (Smythe *et al.*, 1994) using intact A431 cells, which showed that annexin VI is not essential for clathrin-dependent endocytosis. Thus, endocytosis occurs normally in these cells in the absence of annexin VI, as it is not expressed in this cell line. These cells internalized the transferrin receptor via clathrin-coated pits without any apparent requirement for annexin VI. Following stable transfection of annexin VI into these cells, no difference was observed with respect to the qualitative or quantitative aspects of endocytosis of this receptor. Heterologous expression of annexin VI in these cells would at least be expected to have an effect on the rate of endocytosis of the transferrin receptor if it was an essential part of the machinery for vesicle budding. There are several possible reasons for the discrepancy between this work and the work of Lin *et al.* (Lin *et al.*, 1992). There could be functional redundancy between annexin VI and other annexins in A431 cells, such redundancy is already well-established for the anticoagulant and PLA_2 inhibitory activities of annexins. Another possibility is that the assay conditions employed by Lin *et al.* endowed annexin VI with properties that it would not normally have in intact cells (Smythe *et al.*, 1994).

Annexins II and VI have been identified on three types of clathrin-coated vesicles. Annexin VI is only ever found associated with the membrane of the vesicle, whereas annexin II may associate with either the membrane or the coat depending on the vesicle population that is being observed. Annexin II was reported to bind to a 200kDa protein in all of the coated vesicle sub-types while annexin VI apparently bound to a 100kDa protein only in large and small coated vesicles. This protein was identified as dynamin, the GTPase required for secession of invaginating

vesicles (Hinshaw and Schmid, 1995). In fibroblasts endosomes formed without annexin VI are unable to move to fuse with late endosomes and are therefore not degraded. Dynactin is thought to be the physical link between spectrin that is bound to vesicles and dynein which is bound to microtubules. This inability to move towards late endosomes may be due to them lacking spectrin and not being able to link to dynein on microtubules via dynactin (Holleran *et al.*, 1996).

On large endosomes annexin VI and dynamin were reported to be tightly associated while on small endosomes they are both present but without any interaction. It may be that only the large vesicles are able to support this association (Warnock *et al.*, 1996) or alternatively, there may be an unidentified protein containing an SH3 domain, that forms a "bridge" to connect these two proteins together (Shpetner *et al.*, 1996). Experiments by Turpin *et al.* (Turpin *et al.*, 1998) have shown that after dissociation of clathrin annexins II and VI remain associated with the membrane and cannot be removed by either chelation of Ca^{2+} and/or ATP, indicating that the association is Ca^{2+} -independent. This may be an example of annexins initially binding to membranes in a Ca^{2+} -dependent manner, but then becoming bound Ca^{2+} -independently either by stabilization through binding to a membrane component by a Ca^{2+} -independent mechanism (as has been suggested for the binding of annexin II to early endosomes (Jost *et al.*, 1997) or by membrane insertion (see section 2). As both annexins were found with small, medium and large clathrin coated endosomes, they could be part of the general vesicle trafficking machinery rather than being involved in more restricted steps (Turpin *et al.*, 1998).

Recent studies on human fibroblasts have shown that annexin VI bound to the amino-terminal domain of β -spectrin (Watanabe *et al.*, 1994) is as effective as free cytosolic annexin VI at aiding budding of coated vesicles from the membrane (Kamal *et al.*, 1998). When annexin VI is bound in this way to spectrin, spectrin is no longer able to cross-link actin (Watanabe *et al.*, 1994). During exocytosis in chromaffin cells there is depolymerization of F-actin within the submembraneous cytoskeleton, and spectrin becomes reorganized into small areas on the membrane (Aunis and Bader, 1988). Kamal *et al.* (1998) found that during annexin VI-dependent coated pit budding approximately 50% of spectrin from the membrane was lost, and that the whole process could be stopped by using N-acetyl-leucyl-leucyl-norleucinal (ALLN), a cysteine protease inhibitor. In fibroblasts the cells recover one hour after treatment with ALLN and are once again able to internalize cell surface receptors, but the endosomes formed are not able to follow their usual route and are therefore not degraded. Under these conditions new pits are formed at the cell membrane along with the original ones that are no longer able to invaginate, and the new pits are not

associated with spectrin and do not require annexin VI for budding. So it appears that there are two types of coated vesicle budding, one that requires the activation of a cysteine protease to release clathrin from membrane-bound spectrin during budding, and another that is independent of this process (Kamal *et al.*, 1998). In this study annexin VI seems to play a more important role in guiding the endosomes to their correct location rather than for pit budding. It is also possible that annexin VI becomes coupled with a protease that is needed to release the spectrin and thus the annexin VI targets it to the area of membrane where it is needed for coated pit budding. Spectrin is not found on all membranes where coated pits are formed, instead other parts of the cytoskeleton play a part, an example being on the apical surface of polarized epithelial cells where there is virtually no spectrin. When actin microfilaments become depolymerized pit budding is inhibited on the apical membrane, but the spectrin covered basolateral membrane is still able to form endosomes (Gottlieb *et al.*, 1993). These results tend to eliminate annexin VI from having a role in the budding of clathrin coated pits, but instead point to a role for annexin VI in an endocytic pathway that is presumably dispensable in A431 cells.

3.2. Annexin II

Annexin II exists in many subcellular locations depending on cell type and expression of its intracellular ligand p11. There is almost invariably a pool of annexin II at the plasma membrane, it may be expressed on the cell surface, it is also enriched on early endosomes, it has been reported to be present in coated pits/vesicles by immunogold labelling of cryosections, and there are conflicting data regarding the association of annexin II with caveolae (Gruenberg and Emans, 1993). The submembraneous pool of annexin II that exists in a stable association with the actin cytoskeleton acquires this localization by forming a heterotetramer with p11 (Gerke and Weber, 1985). As discussed earlier in the context of exocytosis, binding of p11 to annexin II increases the affinity of annexin II for Ca^{2+} and phospholipids (Powell and Glenney, 1987). Monomeric annexin II is invariably cytosolic but binding to p11 in the tetrameric configuration seems to target the complex to the cytoskeleton (Thiel *et al.*, 1992). In most cells it appears that annexin II is present in molar excess over p11. Because the affinity of annexin II for p11 is high, all the cellular p11 becomes recruited into the complex and the cytosolic pool of monomeric annexin II simply corresponds to the intracellular molar excess. Annexin II is an *in vivo* substrate for PKC (which phosphorylates annexin II on serine-25) (Gould *et al.*, 1986) and the transforming gene product of the Rous sarcoma virus (which phosphorylates annexin II on tyrosine-23) (Glenney, 1986). Phosphorylation of

annexin II at these sites can interfere with the ability of annexin II to aggregate phospholipid vesicles (Johnstone *et al.*, 1992).

The first clear evidence that annexin II might be involved in endocytosis came from experiments that showed annexin II to be one of five major proteins efficiently transferred between the membranes of early endosomes (Emans *et al.*, 1993). No transfer was observed between early and late endosomes as would be expected, as these two compartments do not fuse directly with one another *in vivo* (Gorvel *et al.*, 1991; Gruenberg and Howell, 1989). One of the proteins found to be transferred with annexin II was its light chain, p11, which behaved as a peripheral protein. Contrary to initial expectations, the association between annexin II and early endosomes was found to be Ca^{2+} -independent. Thus, Ca^{2+} chelators have no inhibitory effect on endosome fusion (Wessling Resnick and Braell, 1990) nor do they elute annexin II from the endosomal membrane (Jost *et al.*, 1997). Further evidence supporting a role for annexin II in endocytosis came from experiments (Harder and Gerke, 1993) on Madin-Darby-Canine-Kidney (MDCK) cells, in which a trans-dominant mutant of annexin II induced aggregation of endogenous annexin II and p11. Early endosomes were found to translocate to the region of these aggregates but no other types of vesicles from the endocytic pathway did so. As this association is highly specific this raises the possibility that annexin II has a function related to the earliest steps of the endocytic pathway. In polarized cells the early endosomes are located with the cortical cytoskeleton. Annexin II is bound to the cytoskeleton with α -actinin, moesin, ezrin and actin. In the presence of a low Ca^{2+} level that leads to a disintegration of the cortical cytoskeleton (Nelson and Veshnock, 1987), early endosomes and the annexin II/p11₂ complex move into the cytoplasm (Bomsel *et al.*, 1989; Parton *et al.*, 1989).

Neither mutation of the p11-binding site nor the Ca^{2+} -binding sites in annexin II, prevented the association of annexin II with endosomal membranes (Seemann *et al.*, 1996). These results implied that the domain of annexin II responsible for Ca^{2+} -independent association with endosomes must reside elsewhere in the protein. This was confirmed by mutagenic studies that resolved the endosomal binding site to residues 15 to 24 in the N-terminus, removal of this domain results in failure of annexin II to bind Ca^{2+} -independently with endosomes (Seemann *et al.*, 1996). Note that this domain contains the sites for phosphorylation by PKC and pp60^{v-src} (see above). Clearly, identification of the endosomal receptor for annexin II would greatly enhance our understanding of the role of annexin II in early endocytic events. Other groups have reported that in addition to the Ca^{2+} -independently bound pool of annexin II, there is also a fraction of annexin II that binds Ca^{2+} -dependently to endosomes (Mayorga *et al.*, 1994). This latter pool likely requires intact Ca^{2+} -binding sites for the interaction to

occur (Drust and Creutz, 1988; Glenney, 1986). What is also clear is that neither p11-binding nor complex formation are required for annexin II to bind to endosomes, whereas both Ca^{2+} and p11 are essential for targeting of annexin II to the cytoskeleton (Jost *et al.*, 1994). One possible model for annexin II behaviour in this context is that following engagement of a receptor by ligand, the rapid rise in cytosolic Ca^{2+} drives annexin II to the plasma membrane. At first, during the period of elevated Ca^{2+} , the interaction of annexin II with membranes conforms to the classical view of Ca^{2+} -dependent phospholipid-binding by annexins. However, in regions of receptor internalization, annexin II would also form a stable association with a protein or lipid component of newly formed endosomes. As this secondary interaction is Ca^{2+} -independent annexin II would remain in the endocytic compartment even after cytosolic Ca^{2+} levels return to baseline. There have also been reports of annexin II associated with p11 as a heterotetramer being involved in the fusion of endosomes (Harder and Gerke, 1993). It has also been suggested that annexin II bound to the endosome in a Ca^{2+} -independent manner, in the absence of p11, could easily become fusogenic by binding p11 following clathrin depolymerization and prior to the formation of an early endosome.

Mayora *et al.* (1994) have shown that the combination of arachidonic acid and purified annexin II can cause Ca^{2+} -dependent fusion of endosomes that have previously been washed with EDTA in a cell free system. At micromolar concentrations of Ca^{2+} , endosomes are still able to fuse with each other. Fusion of endosomes was found to be inhibited half maximally by the addition of an annexin II antibody. So there appear to be at least two mechanisms by which endosomes fuse, one which is Ca^{2+} -independent and the other which is Ca^{2+} -dependent, and may involve annexin II (Mayorga *et al.*, 1994). There may be an advantage to the cell in having these different mechanisms, perhaps in determining the eventual destination of the endocytic vesicle, as during phagocytosis it has been observed that phagosomes can fuse directly with lysosomes in the presence of Ca^{2+} (Jaconi *et al.*, 1990). This may also be important in regulating the different endocytic routes taken by endosomes depending on which receptor has been used for internalization (Joiner *et al.*, 1989).

3.3. Annexin I

Like annexin II, annexin I has been found localized with early endosomes (Seemann *et al.*, 1996) although in the latter case the association is Ca^{2+} -dependent. It is particularly interesting that during internalization of activated EGF receptors, up to 38% of the total cellular annexin I (Pol *et al.*, 1997) becomes concentrated with the receptors in multivesicular bodies

and that it is here that phosphorylation of annexin I on tyrosine occurs (Futter *et al.*, 1993). One context in which annexin I was first discovered was as a major substrate for phosphorylation on tyrosine by the EGF receptor (Haigler *et al.*, 1987; Fava and Cohen, 1984). A puzzling aspect of these early observations was that annexin I became maximally phosphorylated some 5 to 10 minutes after engagement of the EGF receptor, considerably later than most other substrates (Wada *et al.*, 1992), but at a time which is now known to correspond to when both proteins are co-localized in the multivesicular body. Phosphorylation of annexin I in this context seems to alter the nature of its association with endosomal membranes. Futter *et al.* (1993) showed that prior to phosphorylation, annexin I appears to bind Ca^{2+} -Independently to the same membraneous compartment as that which contains the EGF receptor, but that following phosphorylation the annexin I-membrane interaction becomes Ca^{2+} -dependent. These observations probably explain the earlier findings of Sheets and co-workers (Sheets *et al.*, 1987) who also described complex phosphorylation-dependent interactions between annexin I and membranes but without any clear mechanism.

Consistent with a role similar to that for annexin II, annexin I is able to aggregate vesicles (Ernst *et al.*, 1991) and may therefore participate in the trafficking of endosomes through the multivesicular body. In another parallel with annexin II, annexin I forms a heteromeric complex *in vivo* with a member of the S100 family, namely S100C, and this complex has been localized to early endosomal membranes (Seemann *et al.*, 1997). S100C is unable to bind endosomal membranes in the absence of annexin I. At present there is very little known about the relevance of the interaction of annexin I and S100C (Seemann *et al.*, 1997) except that it only forms in the presence of Ca^{2+} . Mutational studies of annexin I have shown the N-terminus to be crucial in specifying the localization of the protein in the endocytic pathway. Truncation of the N-terminal 26 residues, but not the N-terminal 13 residues that contain the S100C-binding site, leads to annexin I being localized on late and multivesicular endosomes rather than on early endosomes (Seemann *et al.*, 1996). The region between residues 13–26 that appears to be central in targeting annexin I to early endosomes also contains the sites for phosphorylation by the EGF receptor kinase and PKC. It is probably relevant that when tyrosine-21 is phosphorylated by the EGF receptor kinase, annexin I becomes more susceptible to proteolytic attack which removes part of the N terminus (Haigler *et al.*, 1987; Chuah and Pallen, 1989).

The intrinsic tyrosine kinase activity of the EGF receptor was initially thought to be necessary for lysosomal sorting (Felder *et al.*, 1990), although it has been suggested that this is due to a reduced rate of internalization

rather than a true sorting effect (Ernst *et al.*, 1991; Glenney *et al.*, 1988). However, this is now known not to be the case, as experiments performed using saturating concentrations of EGF showed the rate of internalization of both control and a kinase-dead EGF receptor mutant to be the same (Felder *et al.*, 1990). It is in the multivesicular body that the pathways of the control and mutant kinases diverge. This has also been shown for another growth factor receptor, colony stimulating factor 1 (Carlberg *et al.*, 1991). The EGF receptor kinase is known to be active in the multivesicular body, as EGF stimulation causes a prolonged phosphorylation of the receptor (Nesterov *et al.*, 1990), and it has been shown to be more highly phosphorylated in endosomes than at the plasma membrane (Wada *et al.*, 1992). In multivesicular bodies incubated with [γ - 32 P]ATP the two major proteins that are phosphorylated are annexin I and the receptor itself. This is not seen if the kinase-dead EGF receptor mutant is used (Futter *et al.*, 1993). In this assay annexin I is almost certainly phosphorylated directly by the kinase, as has been established using purified EGF receptor (De *et al.*, 1986). Addition of purified annexin I to partially purified vesicles has shown that annexin I becomes phosphorylated by the EGF receptor kinase in a Ca^{2+} -dependent manner (Cohen and Fava, 1985). It has also been shown that the Ca^{2+} -independent form of annexin I can be phosphorylated by the EGF receptor kinase, turning it into a form that requires Ca^{2+} for association with membranes (Futter *et al.*, 1993), as discussed above. On the basis of these observations it has been proposed that phosphorylation of annexin I causes its release from endocytic membranes and allows inward vesiculation and downstream processing of the multivesicular body (Futter *et al.*, 1993).

4. PHAGOCYTOSIS

Phagocytosis is a specialized form of endocytosis where large particles such as cell debris or microorganisms are ingested into large endocytic vesicles termed phagosomes. Protozoa use this mechanism as a method of feeding but phagocytosis in multicellular organisms serves a different purpose. In mammals phagocytosis is mainly the task of macrophages and neutrophils. These cells protect the body from disease by ingesting invading microorganisms and clear up cell debris and damaged, apoptotic or senescent cells. As a particle is taken into the cell a phagosome forms around it. The size of the phagosome depends on the size of the particle. The phagosome then moves into the cell interior and fuses with a lysosome to form a phagolysosome, where the particle is degraded by lysosomal enzymes. Some of the peptides produced are returned to the cell surface along with recycled proteins of the major histocompatibility complex. The

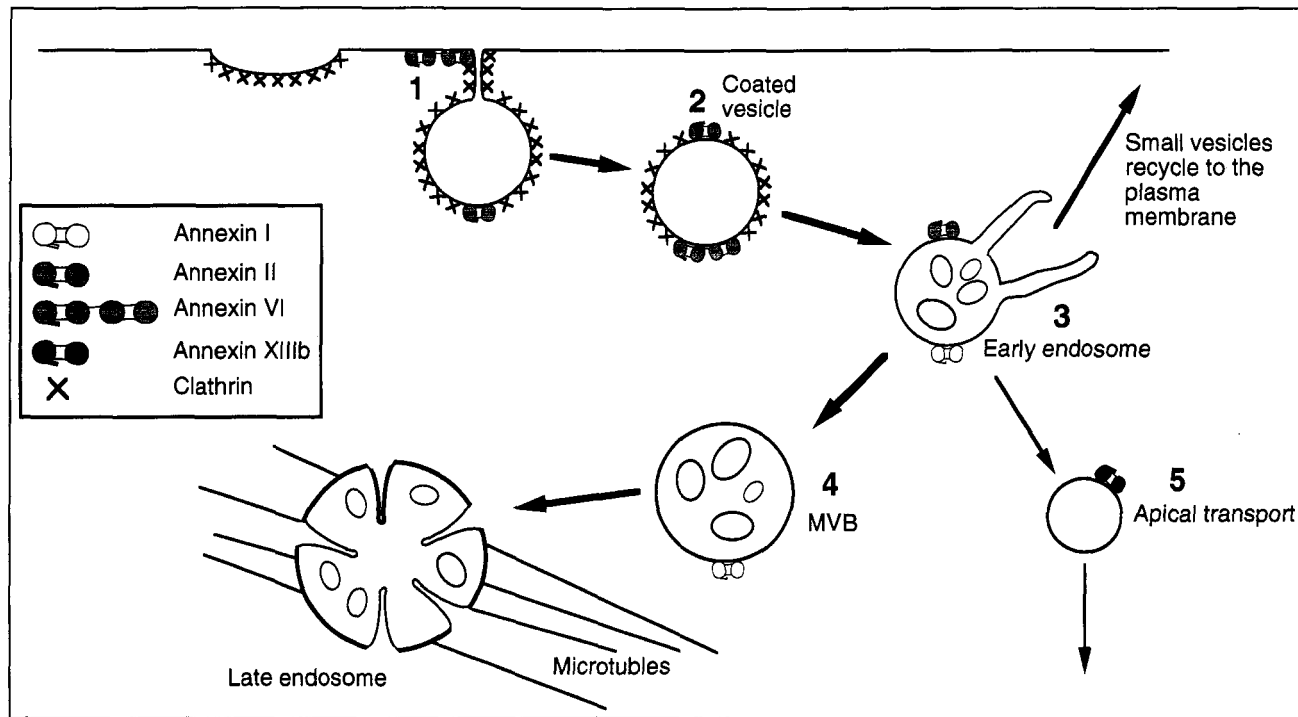


FIGURE 7. Interactions of annexins with the endocytic pathway. Annexins II and VI associate with the forming vesicle (1, 2). Annexin VI is implicated as having a role in severing the membrane stalk (1) and as having an influence in directing the vesicle towards early endosomes (2), whilst annexin II is also found on early endosomes where it is thought to be involved in membrane fusion (3). Annexin I is found localized with early endosomes and MVBs and upon becoming phosphorylated in MVBs it leads to inward vesiculation and downstream processing of this compartment (3,4). Annexin XIIIb is found to associate with vesicles involved in apical transport.

phagosomes mature by a progression of fusion and fission events that are regulated and directional, with certain combinations that are permitted and others that are not. For example, an early endosome and a lysosome would not be allowed to fuse together (Mayorga *et al.*, 1991). For a particle to be ingested it must first bind to the surface of the phagocytic cell, antibodies that have bound to microorganisms are recognised by receptors on the cell surface. Binding of antibody to macrophage or neutrophil receptors induces the cell to engulf the attached particle and form a phagosome.

4.1. Association of Annexins with Phagosomes

Most studies of annexins in this context have focused on neutrophils, with some additional documentation regarding annexins in dendritic cells. Phagocytosis in neutrophils is accompanied by degranulation, caused by fusion of either phagosomes or the plasma membrane with cytoplasmic granules. Degranulation is discussed earlier in section 2. Dendritic cells express annexins I, III, IV, V and VI with expression higher in immature than mature dendritic cells (Larsson *et al.*, 1995). The localization of dendritic cell annexins to the plasma membrane could be a consequence of their being deposited there following exocytosis, or they may be targeted to this site in readiness for some role in phagocytosis (Larsson *et al.*, 1997). But in the absence of any clear data derived from experiments using living cells, it is equally likely that these annexins could participate in a cellular activity completely unrelated to either endocytosis or exocytosis.

During phagocytosis of *Mycobacterium tuberculosis* (the H37Ra attenuated strain) in human neutrophils, Majeed *et al.* (1998) observed that annexins 111, IV and VI translocated from the cytoplasm to the vicinity of phagosomes, whilst no change was seen in the localization of annexins I or V. Annexin IV translocation occurred both in Ca^{2+} depleted neutrophils as well as neutrophils cultured in the absence of external Ca^{2+} . During external binding and envelopment of H37Ra there is an increase in $[\text{Ca}^{2+}]$, which most likely is an important part of the translocation of these annexins. Depletion of intracellular Ca^{2+} in neutrophils allows more of the invading mycobacteria to survive showing that Ca^{2+} -dependent activities are an important part of the degradative process. The association of these annexins to the membranes of phagosomes appears to promote fusion of these vesicles with azurophilic and specific granules. These intracellular fusion events are essential for the killing and digestion of microorganisms.

The relationship between annexins and phagosomes may depend on the contents of, or the type of phagosome, suggesting that neutrophils may have the capacity to regulate and diminish the contents of their phagosomes. Thus, Larsson *et al.* (Larsson *et al.*, 1997) followed the uptake of

Chlamydia trachomatis serovar L2 by receptor-mediated endocytosis in human dendritic cells and compared this with macropinocytic internalization of denitrophenylated human serum albumin (DNP-HSA). Annexin V translocated to DNP-HSA containing endosomes, while annexin III redistributed to vesicles containing *C. trachomatis*. Considering these findings in the light of studies on the uptake of H37Ra, there would appear to be several distinct routes of entry for incoming microorganisms, that probably share certain attributes but which also probably have important differences.

When 5774 macrophages internalize latex beads, either with or without opsonized IgG, annexins I, II, III and V relocate to early endosomes, whereas annexin V is the only one of this group that continues on to the late endosomal compartment (Figure 8). The amounts of annexins I, II, III and V detected in association with phagosomes remained more or less constant during phagosome formation, whilst the amount of annexin IV was found to be significantly higher on older phagosomes. Using a Ca^{2+} chelator, annexins I, II, III, IV and V were all elutable from phagosomes but, with the exception of annexin III, remained membrane-bound at low Ca^{2+} concentrations. Annexin III when removed under the same conditions required a high Ca^{2+} concentration to stay bound to the phagosome (Diakonova *et al.*, 1997). Annexin II, which binds Ca^{2+} -independently to endosomes (Jost *et al.*, 1997) remains at a constant level on phagosomes up to 24 hours following ingestion of latex beads, while phagosomal annexin VI is at higher levels after 24 hours than at two hours after formation (Desjardins *et al.*, 1994).

4.2. Annexin I and Phagocytosis in Neutrophils and Macrophages

Annexin I has been suggested to have an important role in endocytosis in neutrophils. *In vitro*, annexin I increases the rate of vesicle aggregation but does not have a direct effect on the rate of the vesicle fusion. Meers *et al.* (1992) have demonstrated this by using an antibody to the N-terminus of annexin I, which causes an inhibition of vesicle aggregation but has no effect on vesicle fusion. In the absence of annexin I a low level residual Ca^{2+} -dependent fusion can take place which is not inhibited by annexin I antibodies. In the same study, a small amount of annexin I appeared to be involved in linking vesicles together, but this was not transferred from one vesicle to another. These observations led to the suggestion that annexin I monomers are capable of simultaneously being in contact with two separate membranes. These *in vitro* experiments accord well with studies in which annexin I was proposed to have a role in the fusion of phagosomes with lysosomes/endosomes (Emans *et al.*, 1993). During phagocytosis of

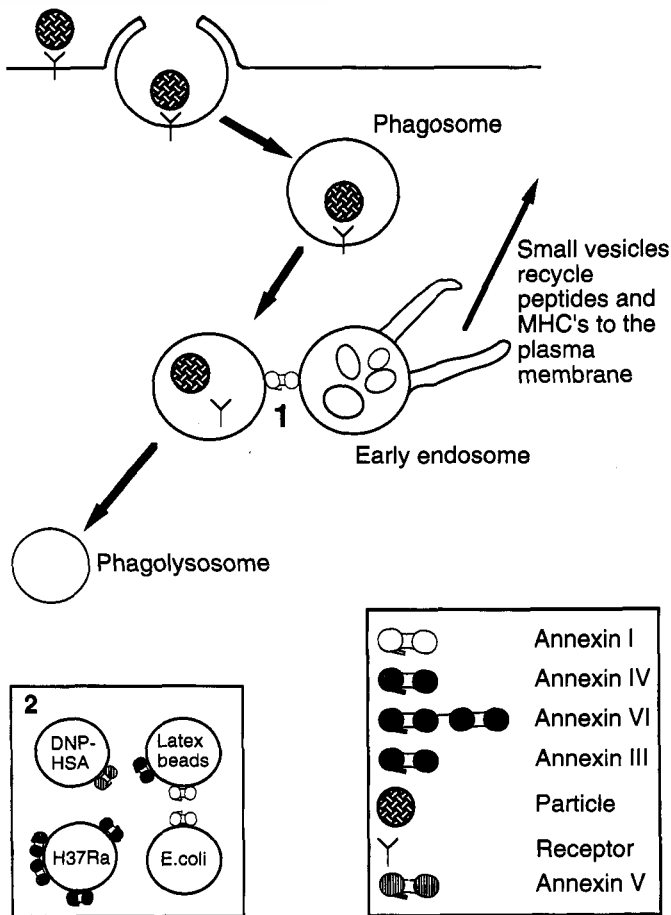


FIGURE 8. Interactions of annexins with the phagocytic pathway. Annexin I is capable of aggregating vesicles as well as being able to physically link them to early endosomes (1). 2 shows examples of annexin association with phagosomes containing a variety of ingested particles. Annexin V is associated with phagosomes containing DNP-HSA (Larsson *et al.*, 1997), annexin I with phagosomes that have ingested *E. coli* (Harricane *et al.*, 1996), annexins III, IV and VI with H37Ra containing phagosomes (Majeed *et al.*, 1998) and annexins I and III when latex beads are ingested (Diakonova *et al.*, 1997).

bakers yeast or *Escherichia coli* in U937 cells that had been induced to undergo differentiation into macrophage-like cells, annexin I which is normally found in patches throughout the cytoplasm, translocates and concentrates around the phagosomes. These observations were not replicated if the cells ingested live *Brucella suis* but were observed if dead *Brucella suis* was ingested (Harricane *et al.*, 1996). Inhibition of lysosome/phagosome

fusion by *Brucella suis* leads to impairment of annexin I translocation (Harmon *et al.*, 1988).

Experiments using macrophages with parasitophorous vacuoles containing *Leishmania mexicana*, revealed similar behaviour for annexin I (Collins *et al.*, 1997). As these vacuoles mature they develop some of the characteristics of the late endosomal compartment, most probably by fusing with vesicles of the endocytic pathway and thereby acquiring late endosomal markers (Prina *et al.*, 1990). Phagosomes containing zymosan or live *Listeria monocytogenes* can fuse with these vesicles but phagosomes containing latex beads or heat-killed *Listeria monocytogenes* failed to fuse with the parasitophorous vacuoles. Similar experiments on *Leishmania amazonensis* yielded similar results (Veras *et al.*, 1992). This inability to fuse correlated with the acquisition of annexin I on the phagosome membrane. It is suggested that acquisition of other membrane constituents like annexin I may directly effect the fusion pathway during phagosome maturation.

During these experiments (Collins *et al.*, 1997) it was shown that the receptor which mediates entry into the macrophage does not appear to have a governing affect on the fate of the vesicle, as use of specific IgG or opsonization with complement components did not enable the vesicle to overcome exclusion when containing heat killed *Listeria monocytogenes*. However, it is interesting to note that if the *Listeria monocytogenes* is killed after ingestion then the phagosome is still able to fuse with the parasitophorous vacuole. Annexin I has previously been implicated in the targeting of vesicles to lysosomes (Futter *et al.*, 1993) so in the phagocytic pathway in macrophages, it is possible that the failure of vesicles containing heat-killed *Listeria monocytogenes* to fuse with the parasitophorous vacuole, is due to annexin I-mediated direct fusion with the lysosomes. Another possibility is that acquisition of annexin I prevents any further fusion reactions (Rohrer *et al.*, 1996). An additional complicating factor is that annexin I, or at least a pool of annexin I, becomes tyrosine phosphorylated on phagosomes containing heat killed *Listeria monocytogenes*. Tyrosine phosphorylation may decrease the ability of annexin I to mediate phagosome fusion with other vesicles (Wang and Creutz, 1994).

As described earlier, neutrophil annexin I translocates from the cytosol to the plasma membrane following administration of opsonized zymosan. After ingestion, a pool of annexin I relocates to the phagosomes. This translocation is probably due to the associated rise in $[Ca^{2+}]_i$ although elevation of intracellular Ca^{2+} concentration elicited by ionomycin is not capable of causing translocation, nor does annexin I become phosphorylated in response to ionomycin. Collectively, these data suggest that annexin I has some role in phagosome function. The mechanism that regulates the association of annexin I with phagosomes is not clear but it clearly depends on signaling factors other than Ca^{2+} alone (Kaufman *et al.*, 1996).

4.3. The Annexin Family in Neutrophil Phagocytosis

Annexin XI is normally located in the cytosol of neutrophils. As just described for annexin I, annexins III and XI translocate to a periphagosomal region during phagocytosis (Sjolin *et al.*, 1997; Ernst, 1991). Translocation of annexin XI requires a rise in intracellular Ca^{2+} levels (Sjolin and Dahlgren, 1996a). It is interesting to note that annexin XI isolated from neutrophils that have been stimulated to phagocytose is truncated, in that it lacks the N-terminal region of the molecule (Sjolin and Dahlgren, 1996a). This processing of the N-terminus is likely to be due to proteolytic enzymes that are associated with phagocytic granules, as EGTA extracts of specific granules contain greater amounts of this cleaved form than does the cytosol. Likewise, limited proteolysis is responsible for the appearance of an N-terminally clipped form of annexin I, which is known to have different biological properties to the holo-protein (Sjolin and Dahlgren, 1996b; Chuah and Pallen, 1989). Furthermore, annexin XI contains a binding site in its N-terminus for calcyclin (Watanabe *et al.*, 1994), another example of an interaction between an annexin and a protein of the S100 family, that may be regulated by proteolytic cleavage of the N-terminus.

As with other examples of phagocytosis, internalization of yeast by neutrophils leads to translocation of annexins I and III to the area of the phagosome (Kaufman *et al.*, 1996; Ernst, 1991). When cells are invaded by *Chlamydia trachomatis*, which is not the same as true phagocytosis (La Verda and Byrne, 1994), annexins III, IV and V, but not annexins I or VI, move to the region of the vacuole enclosing this yeast (Majeed *et al.*, 1994). Taken together with results on the internalization of *Brucella suis* described earlier, the redistribution of annexins in phagocytosing cells seems to depend at least in part on what is ingested. Work by Ernst *et al.* (1991) has shown intracellular redistribution of annexin III in neutrophils ingesting opsonized yeast. This annexin concentrates around phagosomes as was also reported in the phagocytosis of H37Ra. Annexin III is extremely abundant in this cell type, representing more than 1% of the total protein content of the cell. The concentration around the phagosome was observed by laser scanning confocal microscopy to be nine times greater than that in the cytoplasm and persisted for more than an hour after the phagocytic event. The translocation of annexin III is not a general property of Ca^{2+} -binding proteins or cytosolic proteins as calmodulin did not show a similar behaviour. Moreover, given that the binding of annexins to pure phospholipids is fully and rapidly reversible on addition of a Ca^{2+} chelator (Meers, 1990; Pepinsky *et al.*, 1988), the fact that annexin III persists around the phagosome indicates that it is not solely dependent on a local elevated Ca^{2+} concentration. Annexin III may therefore have a

specialized role in structural and metabolic events associated with phagocytosis.

5. ANNEXINS IN REGULATED EXOCYTOSIS

It is obvious from the previous section that there are many potential roles for annexins in endocytosis. Although most of the data supporting such roles are derived from *in vitro* experiments, an increasing number of studies now substantiate the idea that annexins have roles in membrane fusion processes in living cells. In particular, important advances have been made in the investigation of secretory roles for annexins. These studies are the focus of this section.

5.1. Annexins from Simple Organisms

Annexins or annexin-like proteins have been cloned and characterized in protozoans, e.g. in *Giardia* (Morgan and Fernandez, 1995) and *Paramecium* (Knochel *et al.*, 1996). Thus, immunochemical and biochemical techniques led to the identification of several proteins in *Paramecium* that appeared to be annexins. The same antibodies revealed dynamic labelling of cortical structures during trichocyst exocytosis, arguing for an involvement of protist annexins in membrane fusion events. Two annexins, annexin XII and a 40 kDa annexin, were identified in the freshwater cnidarian *Hydra vulgaris* (Schlaepfer *et al.*, 1992). These proteins localize to specific hydra cell types, and were not co-expressed in the same cells. It was argued therefore, that annexin XII and 40 kDa are not implicated in “housekeeping” functions but perform specialized biological roles. Annexin XII was detected in epithelial battery cells and in epithelial cells of the peduncle region. The 40 kDa hydra annexin was predominantly found in the cytoplasm of nematocytes. These cells fire tiny spears in defense in what is an explosive form of exocytosis. This finding may indicate that annexins have acquired roles in exocytosis early in eukaryotic evolution.

5.2. Annexin I in Exocytosis

Studies on the mechanism of insulin secretion in the rat have suggested the possible involvement of annexin I. Immunocytochemical studies have shown that annexin I is strongly expressed in the pancreatic islets, where it co-localizes with insulin-containing granules. The level of annexin I associated with insulin granules is increased in glucose-treated rats when compared to those starved overnight. Moreover, annexin I levels in pancreatic

islets are severely decreased in diabetic rats (Ohnishi *et al.*, 1995). In addition to the long-term effects of glucose on annexin I expression, glucose also induces a dramatic increase of annexin I phosphorylation on serine. A protein kinase C inhibitor (H7) reduces glucose-induced phosphorylation and leads to significantly reduced insulin secretion (Ohnishi *et al.*, 1995). However, the effect of H7 on secretion was not nearly as great as inhibition of PKC-dependent phosphorylation (up to 90%). Annexin I is expressed in several other endocrine cells, including the anterior pituitary (Thurgeon *et al.*, 1991) and the adrenal medulla (Wang and Creutz, 1992; Michener *et al.*, 1986). Although there is no evidence to suggest a role for annexin I in hormone secretion from the anterior pituitary, annexin I has been shown to support the aggregation of isolated chromaffin granules, an activity that is inhibited by phosphorylation of annexin I by protein kinase C (Wang and Creutz, 1992).

5.3. Annexin II in Exocytosis

The first experimental evidence implicating annexins in exocytosis came from studies on annexin II, in which the purified protein was shown to be able to aggregate isolated chromaffin granules in the presence of physiological concentrations of Ca^{2+} . Addition of arachidonic acid led to fusion of the aggregated granules, suggesting a role for annexin II as a Ca^{2+} -dependent secretory mediator (Drust and Creutz, 1988). The notion that annexin II might have a role in secretion gained further credibility when it was shown that addition of purified annexin I to permeabilized chromaffin cells delayed secretory run-down (Ali *et al.*, 1989). Following these early studies bovine adrenal chromaffin cells have become the most extensively used model for the study of annexin II in exocytosis.

In these cells there is a significant pool of monomeric annexin II in the cytosol (Sagot *et al.*, 1997). It has more recently been shown that upon nicotinic stimulation, annexin II translocates from the cytosol to the submembraneous region, where complex formation with p11 (which is restricted to the cortical region even in unstimulated cells) takes place (Koenig *et al.*, 1998; Chasserot Golaz *et al.*, 1996). By binding to both the granule membrane and the plasma membrane, the annexin II₂/p11₂-heterotetramer would be ideally placed to anchor secretory vesicles to the cell cortex. Consistent with this model, a Ca^{2+} rise to 1 μM after nicotinic stimulation was found to be sufficient to induce translocation of approximately 80% of total annexin II to a Triton X-100 insoluble membrane fraction (Sagot *et al.*, 1997). Moreover, the annexin II₂/p11₂ complex was found localized in the cortical cytoskeleton underlying the plasma membrane and associated with

chromaffin granules (Gerke and Moss, 1997). Annexin II has been suggested to form physical connections between the plasma-membrane and the membrane of chromaffin granules (Nakata *et al.*, 1990). High resolution images obtained by cryo-electron microscopy showed that short intergranular or granule-membrane bridges formed only in the presence of annexin II, although in this study the molecular composition of these bridges was not investigated. Nevertheless, such intermembrane junctions could be formed by annexin II molecules bound to the outer leaflet of each vesicle linked by a central p11 dimer (Lambert *et al.*, 1997).

The hypothesis that both annexin II and the annexin II₂/p11₂-heterotetramer are involved in exocytosis is supported by the run-down assays of secretion in permeabilized cells (see below). Stimulation of chromaffin cells also leads to phosphorylation of annexin II by protein kinase C. In vitro, phosphorylation by protein kinase C decreases the granule-binding affinity of annexin II, induces disassembly of the heterotetramer and leads to granule fusion (Regnoui *et al.*, 1995). Moreover, Sarafian *et al.* (1991) showed that prior phosphorylation of annexin II by PKC was required to endow annexin II with maximal capacity to arrest secretory run-down in permeabilized chromaffin cells. Also, a synthetic annexin II peptide corresponding to amino acids 15–26 (a region of the N-terminal domain containing phosphorylation sites but not the p11-binding site) blocks the nicotine-induced translocation of annexin II to the cell periphery and inhibits Ca²⁺-triggered catecholamine secretion (Chasserot Golaz *et al.*, 1996). It is possible therefore that N-terminal phosphorylation regulates translocation of annexin II from the cytosol to the plasma membrane.

Using membrane capacitance tracing to follow exocytotic fusion events, Koenig *et al.* (1998) recently discovered that disruption of preformed annexin II₂/p11₂ complexes in bovine pulmonary artery endothelial cells inhibits regulated exocytosis. This was demonstrated by loading the cells with a synthetic peptide comprising the N-terminal 14 residues of annexin II. The excess of free peptide displaces the endogenous annexin II by competing for binding sites on p11. One interpretation of these data is that the monomeric annexin II liberated in this way inhibits exocytosis, but given the weight of data supporting a role for annexin II in exocytosis, the more likely explanation is that the annexin II₂/p11₂ complex has a pro-exocytotic role. The role of annexin II in Ca²⁺-regulated secretion is thus not restricted to chromaffin cells (Gerke and Moss, 1997). For example, Ca²⁺-induced exocytosis of lamellar bodies in permeabilized alveolar epithelial cells, is stimulated specifically by monomeric annexin II and the annexin II heterotetramer, since in the same assay, annexin I, III, IV, V and VI were ineffective (Liu *et al.*, 1996).

5.4. Annexin III in Exocytosis

There are relatively few accounts of annexin III involvement in exocytosis. Yet given the abundance of this protein in neutrophils it is reasonable to suppose that it may participate either in the phagocytic (discussed earlier in section 4.3) or secretory activities of these cells. In neutrophils, permeabilization causes leakage of almost the entire cellular pools of annexins V and VI, whereas almost half the total pool of annexin I and 12% of annexin III are retained, presumably due to association with granule membranes. Under the same experimental conditions secretion falls to about 80% the maximal value of control cells. Subsequent re-addition of annexins I and III increases secretion by up to 15% and 90% respectively (Rosales and Ernst, 1997). The reasons for the dramatic effects of annexin III, in comparison to annexin I, are not known.

5.5. Annexin V in Exocytosis

As for annexin III, there is little information linking annexin V to a role in exocytosis. In the rat central nervous system annexin V is preferentially localized to axon terminals, with the reactivity associated with synaptic vesicles and mostly co-labelled with that for synaptophysin. Cluster formation of purified synaptic vesicles was dependent on the presence of endogenous or human annexin V and Ca^{2+} (Gotow *et al.*, 1996). Annexin V only aggregates the vesicles, but does not induce vesicle fusion. In the axon terminal synaptic vesicles are linked to cytoskeletal elements and to each other via synapsin I (Hirokawa *et al.*, 1989). This linkage is visible in electron microscopy as thin filamentous strands. Upon stimulation and Ca^{2+} influx, activation of a Ca^{2+} /calmodulin-dependent kinase leads to phosphorylation of synapsin I, which causes detachment of synapsin I from the vesicle membrane. The vesicles are then free to move closer towards the synaptic membrane and may there become closely attached by annexin V. It was hypothesized therefore, that neuronal annexin V serves to induce Ca^{2+} -dependent clustering of vesicles close to active zones, thereby contributing to the efficiency of synaptic transmission (Gotow *et al.*, 1996). Annexin V is highly expressed in atrial myocytes. These cells secrete atrial natriuretic peptide, which is normally stored in atrial secretory granules. Annexin V was one of only a few proteins (that included annexin VI) that bound Ca^{2+} -dependently to isolated atrial secretory granules (Doubell *et al.*, 1991). Since these cells are stimulated to secrete by elevation of intracellular Ca^{2+} it is possible that annexin V may modulate release of atrial natriuretic peptide.

5.6. Annexin VI in Exocytosis

Annexin VI is widely expressed in secretory tissue types, in some cases localized exclusively to the inner face of the plasma membrane of specialized secretory regions (Jones *et al.*, 1994). In a survey of normal human tissues annexin VI was found to be strongly expressed in some but not all endocrine cells, including the B-cells of the pancreas, enterochromaffin cells in the intestine, Leydig cells in the testis and the ovary (Clark *et al.*, 1991). Annexin VI immunoreactivity was not evident in the parathyroid. It is interesting to note that in the former group of endocrine cells and tissues, secretion is stimulated by a rise in intracellular Ca^{2+} , whereas in parathyroid cells it is a fall in intracellular Ca^{2+} that triggers exocytosis. In addition, annexin VI appears to counteract the pro-secretory activities of other annexins, possibly due to its structural peculiarities. Thus vesicle aggregation and fusion mediated by annexins II and VII, for example, is inhibited by annexin VI (Zaks and Creutz, 1990b).

So could annexin VI be a negative regulator of exocytosis, perhaps by acting as a competitive inhibitor of other annexins in binding to and aggregating vesicles (Donnelly and Moss, 1997)? In support of this idea annexin VI is strongly expressed in non-lactating breast epithelial cells but is down-regulated in lactating breast. This is precisely what would be expected of a protein with potent anti-secretory activity. The presence of annexin VI in other secretory cell types (described above) can be explained if one accepts the idea that secretory cells possess both positive and negative regulators of secretion. This makes good biological sense as a combination of pro- and anti-secretory proteins enables cells to “fine-tune” the secretory response with greater accuracy. This is likely to be important when considering the need to tightly regulate the secretion of extremely potent molecules.

5.7. Annexin VII in Exocytosis

Synexin (annexin VII) binds to and hydrolyzes GTP in a Ca^{2+} -dependent manner. In an *in vitro* model of chromaffin granule fusion, synexin drives the Ca^{2+} -dependent fusion process, where it is further activated by GTP (Caohuy *et al.*, 1996). It was argued therefore that synexin acts as an atypical G protein that is responsible for both detecting and mediating the $\text{GTP}/\text{Ca}^{2+}$ signal in exocytosis. Synexin is one of the most likely candidates to act as a membrane fusogen. This role still needs to be proven *in vivo*. However, there is more compelling *in vitro* evidence for an involvement of annexin VII in lung surfactant secretion. Lung epithelial type II cells synthesize surfactant and store it in lamellar bodies prior to exocytosis into the alveolar lumen. Lung annexin VII was found to induce, in the presence

of Ca^{2+} , aggregation of isolated lamellar bodies, liposomes and isolated plasma membrane fractions, and fusion between lamellar bodies and plasma membranes, but not however fusion between plasma membrane and liposomes (Chander and Wu, 1991). Maximal binding of annexin VII to lamellar bodies as well as to the plasma membrane requires $200\mu\text{M}$ Ca^{2+} , but the Ca^{2+} requirement for binding, aggregation and fusion appears to be reduced by arachidonic acid. A 76 kDa annexin VII-binding protein is present in both lamellar body and plasma membrane and may function as a docking protein in secretion. Therefore, binding of annexin VII to lamellar bodies and to the plasma membrane takes place probably both via phospholipids and protein. Self-association of membrane-bound annexin VII was suggested to be an important aspect of the membrane aggregation and fusion properties of this protein (see the “hydrophobic bridge” hypotheses, section 7). Interestingly, self association of annexin VII in solution, for example by high (1 mM) Ca^{2+} or stilbene disulfonic acids (Liu and Chander, 1995) inhibits its aggregation and fusion activity. From this observation it was argued that self-associated forms of membrane-bound and soluble annexin VII may differ in protein conformation (Sen *et al.*, 1997). This may support the hypotheses that the fusogenic activity of annexins requires a specific, possibly membrane-inserted conformation (as discussed below).

5.8. Annexin XIII in Exocytosis

In non-synaptic secretory cells, secretory granules reside in the cytoplasm and must therefore translocate to the plasma-membrane in order to release their contents. In polarized secretory cells there is the additional requirement that secretion occurs only at the apical membrane. This requires specialized proteins that are able to deliver intracellular secretory vesicles to the correct part of the cell. In MDCK cells annexin XIIIb is implicated in this process. Annexin XIIIb is an N-terminal splice variant of annexin XIII and is the only member of the mammalian annexin family to be N-terminally myristoylated. Unlike other annexins annexin XIIIb therefore has an integral membrane-targeting signal. Both anti-annexin XIIIb antibody and recombinant, unmyristoylated annexin XIIIb inhibited apical delivery of secretory vesicles, providing good evidence for a role for annexin XIIIb in apical vesicular transport (Lafont *et al.*, 1998). Annexin XIIIb is found enriched on vesicles in the apical side of polarized MDCK cells. Vesicle transport to the apical but not to the basolateral plasma membrane is sensitive to antibodies directed against the unique exon in annexin XIIIb, whereas basolateral but not apical vesicle traffic is affected by targeting the SNARE-SNAP-NSF-dependent docking-fusion machinery through the use of antibodies or bacterial toxin (Ikonen *et al.*, 1995). Interestingly, transcytosis of pre-internalized IgA receptor to the apical surface of these cells is

N-ethylmaleimide and Botulinum neurotoxin sensitive, indicating that this transport route is SNARE-SNAP-NSF-dependent. These observations indicate that annexin XIIIb is part of a specialized NSF-independent vesicle transport and fusion pathway, that operates in parallel to the SNARE-SNAP-NSF-dependent exocytotic machinery.

6. EVIDENCE AGAINST A ROLE FOR ANNEXINS IN VESICLE TRAFFICKING

The previous sections provide a weight of evidence that annexins function in vesicle trafficking, but besides these favourable reports, there are data arguing against the involvement of annexins in these processes. The slime mould *Dictyostelium discoideum*, for example, expresses two isoforms (47 and 51 kDa, respectively) of annexin XIV (a distant relative of annexin VII). In gene disruption studies, pinocytosis as well as phagocytosis and other cellular processes were normal in mutants lacking both isoforms of annexin XIV (Doring *et al.*, 1995).

More direct evidence against the involvement of annexins in exocytosis comes from studies showing that elevation or diminution of functional annexin II levels in rat PC12 (rat pheochromocytoma) cells failed to alter Ca^{2+} -triggered dopamine release in a permeabilized cell system (Graham *et al.*, 1997). This argues against a role of annexin II in Ca^{2+} -dependent exocytosis and is in agreement with the observation that exocytotically active neuronal cells contain little annexin II. It is also difficult to find a place for annexins to function given the apparent ubiquitous expression of the SNAP-SNARE exocytotic machinery and the apparent absence of any annexins from this machinery. This is discussed in more detail below. Even in instances where the data appear compelling, such as the claim that annexin VI is required for budding of clathrin coated pits, the results must be squared with observations that endocytosis proceeds normally in cells that lack this protein (Smythe *et al.*, 1994). Indeed, the general well-being of annexin VI knock-out mice recently generated in our laboratory (Hawkins, Roes and Moss, unpublished), provides proof that if annexin VI is involved in either secretory or endocytic processes then at best it has only a minor modulatory role.

7. ANNEXINS: FUSOGENIC OR NON-FUSOGENIC

It is currently unknown whether proteins play a direct role in membrane fusion. SNARE proteins were long thought to exert such a direct role, but recent studies suggest that SNARE complexes disassemble after

docking but prior to the actual fusion step (Tahara *et al.*, 1998). It is possible that proteins are only required to target vesicles to release sites and to bring lipid bilayers close together, and that these then fuse without further involvement of protein. However, spontaneous fusion is an energetically unlikely event. Zheng and Bobich (1998) raised the possibility that formation of the SNARE complex in exocytosis would release sufficient energy to overcome the energy barrier between the fusing membranes. In the final stages of exocytosis the formation of a fusion pore was observed. The fusion pore may be created by localized rupture of a hemifused bilayer. However, current models postulate that the fusion pore is formed by protein. Synaptophysin is perhaps the most attractive candidate to fill this role (Zheng and Bobich, 1998).

Many researchers have come to the conclusion that annexins are not fusogenic themselves, but instead serve to draw membranes together which will then fuse if further perturbed, for example by free fatty acids (Zaks and Creutz, 1990a). However, the following observations suggest that annexins differentially affect aggregation and fusion and indicate that annexins do not promote fusion by their aggregating affect alone: 1. Rates of fusion at different calcium levels are often different from the relative aggregation rates. 2. High annexin concentrations inhibit fusion but not aggregation (Zaks and Creutz, 1990a). 3. In a chromaffin granule assay of aggregation and fusion Regnoui *et al.* (1995) found that only unphosphorylated but not PKC-phosphorylated annexin II induced granule aggregation. On the other hand, PKC-phosphorylated annexin II led to a spectacular induction of granule fusion, whereas unphosphorylated annexin II was without effect. Phosphorylation of annexin II also caused disassembly of the annexin II₂/p11₂-heterotetramer. Therefore, aggregation appears to require the heterotetramer, whereas fusion is induced by monomeric annexin II. These findings are in agreement with a report by Sarafian *et al.* (1991) who in run-down assays of secretion found no restoring effect of annexin II in staurosporine (PKC inhibitor) treated cells, whereas PKC pre-phosphorylated annexin II was effective. 4. Liposomes prepared from acidic phospholipids such as phosphatidylserine, phosphatidic acid and phosphatidylethanolamine, or mixtures of these, fuse directly if calcium and annexin VII are added to them in solution. However, liposomes prepared from phosphatidylinositol are only aggregated by annexin VII (Pollard *et al.*, 1988).

As discussed above, we now have a detailed understanding of how annexins are able to aggregate vesicles. On the other hand, it remains largely unclear how annexins induce membrane fusion. A model to explain annexin driven membrane fusion was suggested Pollard and co-workers for annexin VII and was then called the "hydrophobic bridge" hypothesis

(Figure 9) (Pollard *et al.*, 1992). In this model, Ca^{2+} causes annexin VII to undergo significant conformational changes leading to the exposure of hydrophobic residues with the consequence of polymerization and simultaneous insertion of the annexin VII polymer into two adjacent target membranes (lipidation). Fusion ensues when the “hydrophobic bridge” stabilizes both bilayers and provides a pathway for lipids on facing leaflets of both membranes to cross and mix. In the low dielectric environment annexin VII polymers will then dissociate, thereby providing a hydrophobic pathway for the trans-leaflets to approach. After re-orientation of the trans-leaflets is complete, some or all of the annexin VII molecules are thought to leave the membrane. Several experimental observations are in favour of this model. In artificial phospholipid bilayers annexin VII increases the membrane capacitance and exhibits both gating currents and voltage sensitive Ca^{2+} channel activity (Pollard *et al.*, 1988). This was taken as indication of the ability of annexin VII to penetrate the bilayer (Raynal and Pollard, 1994). Furthermore, electron microscopy studies support the idea that annexin VII molecules self-associate to form dimers, which are then able to further

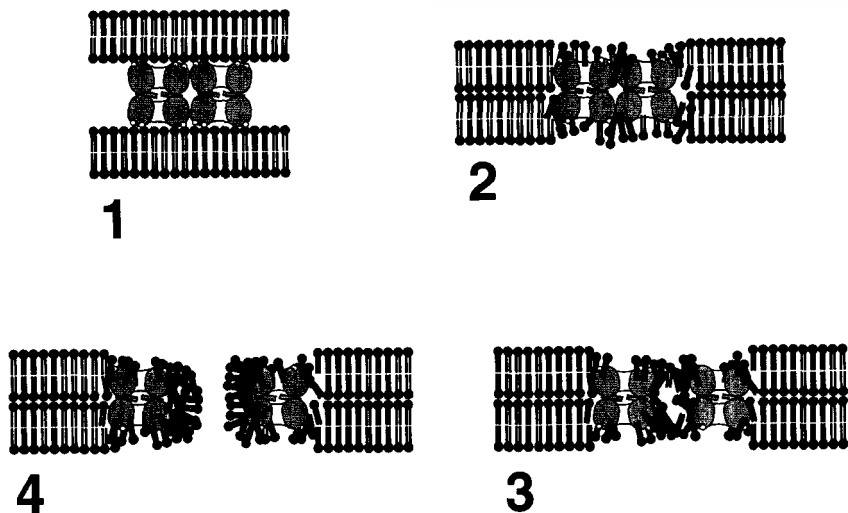


FIGURE 9. The “hydrophobic bridge” hypotheses. 1, a polymer formed of annexin VII dimers links juxtaposed membranes; 2, lipidation occurs from both target membranes, leading to complete insertion of the polymer into the membranes; 3, in the low electric environment of the two bilayers the polymer depolymerizes and allows diffusion of phospholipids along the dimer surface; 4, phospholipids from opposite membrane surfaces can now mix with each other, subsequently leading to membrane fusion (modified from Pollard *et al.* (1992)).

polymerize. Aggregation and fusion activity of annexin VII can be inhibited by induction of self-association of the protein in solution (Liu and Chander, 1995), but this inhibition does not occur when annexin VII spontaneously self-associates on membranes. This may indicate different conformations of self-associated membrane-bound and soluble annexin VII (Sen *et al.*, 1997). The "hydrophobic bridge" model is based on the assumption that annexin VII is able to insert into phospholipid bilayers.

The crystal structure of full-length annexin VII is so far unavailable, although the crystal structure of the C-terminal core of the related annexin from *Dictyostelium discoideum* has been solved (Liemann *et al.*, 1997). However, crystal structures of other annexins with *in vitro* ion channel activity, e.g. annexin V, indicate that these molecules do not insert into membranes, but remain peripherally attached to the plasma membrane inner leaflet in the Ca^{2+} -bound conformation where they tend to form two-dimensional crystalline arrays. According to the "micro-electroporation"-model the membrane-attached annexins allow ion fluxes across the bilayer by exerting a strong electric field which would locally lead to disruption of the phospholipid leaflets adjacent to the protein surface. Obviously, these data question the "hydrophobic bridge" model. More recently, studies by Haigler and his group (Langen *et al.*, 1998b) do point to a membrane-spanning configuration of annexins which is achieved under conditions of low pH. Low pH induces annexin VII to undergo conformational changes which lead to formation of a continuous α -helix from a helix-loop-helix motif in the neutral pH structure. Based on these conformational changes Langen *et al.* (1998b) proposed a model in which the annexin core protein forms seven membrane spanning α -helices. Although intriguing, it remains to be seen whether other annexins are also able to take on this low pH conformation, whether this conformation is solely responsible for ion channel activity and in which physiological environments it occurs. Other recent evidence suggests that annexin I at pH 6.0 and in the absence of Ca^{2+} penetrates a phospholipid monolayer, whereas annexin V lacks this ability (Rosengarth *et al.*, 1998). This would mean that ion channel activity at least of annexin V must be due to membrane "micro-electroporation" or a similar mechanism that does not require membrane insertion of the channel protein. The most important question in this context, is whether the membrane-spanning conformation induced experimentally by low pH plays a role in the fusogenic properties of annexins. It is interesting to note that viral fusion proteins undergo similar conformational changes at low pH (White, 1995). Moreover, some viruses appear to use annexins for fusion with target membranes. Cytomegalovirus appears to fuse with and infect human endothelial cells via annexin II (Wright *et al.*, 1994), whereas the

hepatitis B virus infects human hepatocytes via annexin V (Hertogs *et al.*, 1993). It is possible therefore, that under physiological conditions annexins function as fusion proteins involved in endoplasmic fusion events and due to biochemical similarities can be used by some viruses, instead of their own viral fusion proteins for ectoplasmic fusion.

Regulated exocytosis requires both Ca^{2+} and GTP. Ca^{2+} and GTP-sites of action are thought to be closely associated. Annexin VII binds to and hydrolyzes GTP in a Ca^{2+} -dependent manner and is therefore a likely candidate for a sensor protein in Ca^{2+} /GTP-regulated exocytosis. Binding of GTP upon elevation of Ca^{2+} is thought to switch annexin VII into an "on" state, in which it drives membrane fusion (Pollard *et al.*, 1998). However, the identity of the GTP induced "on" state is unclear. The "on" state may be achieved by GTP-induced conformational changes in annexin VII, but it is unknown whether GTP binding induces any conformational changes and so far, no annexin structure has been solved in the presence of a nucleotide. Therefore it remains to be shown whether conformational changes in annexin VII similar to those obtained with annexin VII under conditions of low pH are involved in its fusogenic properties.

In *in vitro* studies, several annexins have been shown to promote fusion of liposomes (Raynal and Pollard, 1994). Many of these studies use labelled phospholipids to study membrane fusion. The presence of these phospholipids may evoke sufficient membrane perturbation to induce fusion of closely associated membranes. Therefore, fusogenic activity of annexins has to be viewed with caution, because fusion may simply be a passive result of the aggregating properties of annexins, although fluorophore-labelled fusion of artificial liposomes was not observed (Oshry *et al.*, 1991). Secretion from permeabilized type II alveolar epithelial cells is stimulated by monomeric annexin II and the annexin II heterotetramer with similar efficiency (Liu *et al.*, 1996). However, in the same study, the heterotetramer showed much more potency to induce fusion of lamellar bodies with PWPE liposomes. This appears to contradict the notion that only phosphorylated, monomeric annexin II is fusogenic, whereas annexin $\text{II}_2/\text{p11}_2$ only aggregates vesicles. In simple protein granule mixtures annexin $\text{II}_2/\text{p11}_2$ is able to display fusogenic properties, but only in the presence of elevated concentrations of cis-unsaturated fatty acids (Drust and Creutz, 1988). It appears possible that aggregation of vesicles and liposomes is sufficient to induce fusion if phospholipids are perturbed by unsaturated fatty acids or by the presence of labelled phospholipids (Oshry *et al.*, 1991). At 1mM Ca^{2+} annexin $\text{II}_2/\text{p11}_2$ shows four fold greater aggregation activity than monomeric annexin II (Liu *et al.*, 1995), which may explain the differences in fusion levels of lamellar bodies and PS/PE liposomes.

8. CONCLUSION AND OUTLOOK

Many of the properties of proteins involved in NSF, SNAPs, SNAREs-dependent vesicle transport and membrane fusion machinery (Zheng and Bobich, 1998) are shared by annexins. Annexins bind to negatively-charged phospholipids in a Ca^{2+} -dependent manner, as does synaptotagmin. Annexins bind to vesicular membranes (as do synaptobrevin, synaptophysin and synaptotagmin) and the plasma membrane (as do syntaxin and SNAP-25). They bind both to the plasma membrane and cytoskeletal elements (synapsin I). Annexins tend to self association and polymerize (as do synaptotagmin, synaptophysin), bind GTP (as do rabs), bind and bundle F-actin (also shown for synapsin I), are involved in membrane organization (PEP proteins) and form membrane channels (as does synaptophysin), to name but a few parallels. Besides the ubiquity of the NSF, SNAPs, SNAREs-dependent exocytotic machinery, NSF-independent vesicle transport and fusion pathways have been identified (Gerke and Moss, 1997). One such NSF-independent mechanism was shown to involve annexin XIIIb in MDCK cells (Lafont *et al.*, 1998). This raises the possibility, that annexins provide a specialized exocytotic pathway that works in parallel to other mechanisms such as NSF-dependent exocytosis. Alternatively, the interaction of the C-terminus of annexin I with synaptotagmin appears to support

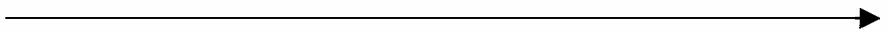
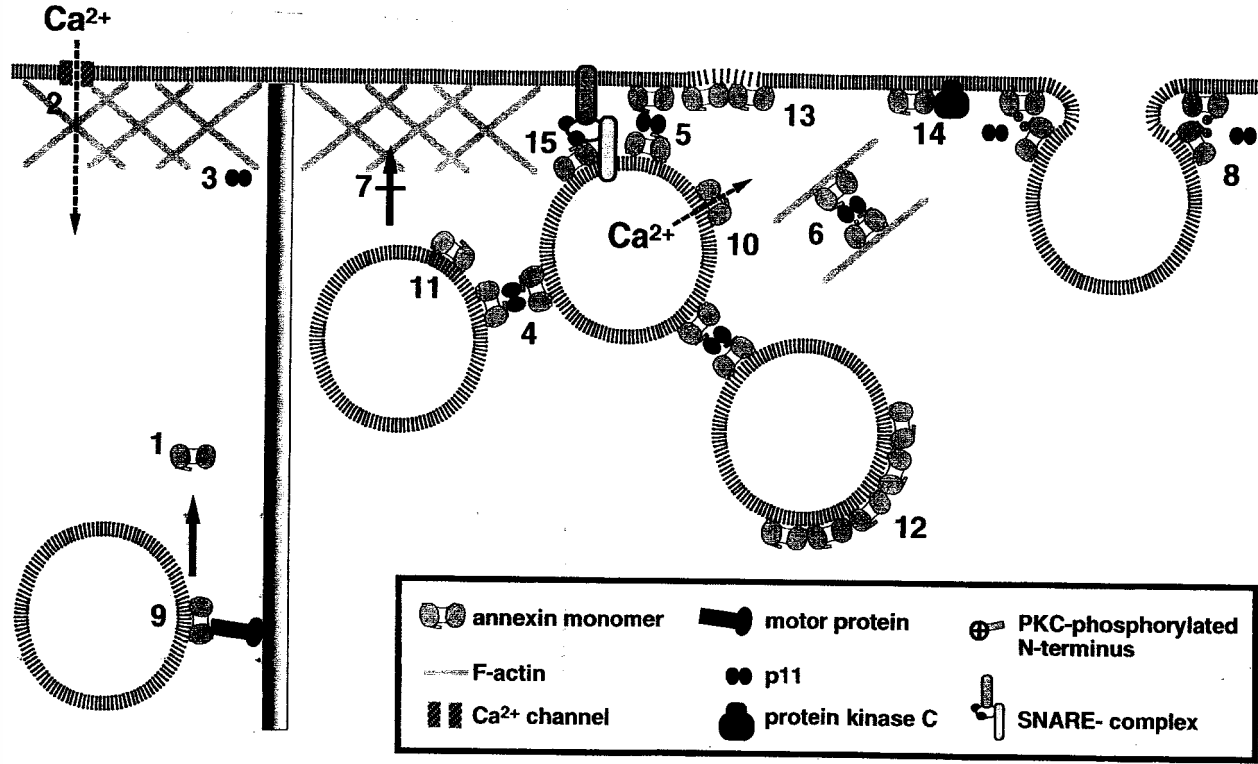


FIGURE 10. Putative roles of annexins in vesicle trafficking and secretion. In unstimulated chromaffin cells, a large fraction of annexin II is present in monomeric form in the cytosol (1). Upon stimulation and rise of intracellular free Ca^{2+} due to Ca^{2+} -influx (2), annexin II translocates to the cell cortex and forms a (Ca^{2+} -independent) heterotetrameric complex with p11 (3), which is already present in the submembraneous area before stimulation. The annexin II/p11₂-heterotetramer crosslinks and thereby aggregates secretory vesicles (4), anchors secretory vesicles to the plasma membrane (3), and causes F-actin bundling (6). The latter leads to disassembly of the cortical actin web, which until then prevented approach of chromaffin granules (7). The granules can now move into close proximity of the plasma membrane. There, protein kinase C phosphorylation of annexin II induces disassembly of the heterotetramer. Phosphorylated annexin II then induces fusion of the chromaffin granule with the plasma membrane (8). Annexins may also be involved in vesicular transport along the cytoskeleton, possibly by forming an adaptor protein for apical transport carriers to motor proteins, as suggested for annexin XIIIb in MDCK cells (Lafont *et al.*, 1998) (9). Annexin VI is localized in the plasma membrane undercoat and upon contact with chromaffin granules releases intragranular Ca^{2+} , thereby augmenting the cytosolic Ca^{2+} signal (10). Furthermore, annexins may be involved in the organization of membrane microdomains of specific lipid composition, e.g. PIP_2 /cholesterol-rich domains (11), stabilization (12) or destabilization of fusion membranes (13), and regulation of PKC (14) and/or elements of the NSF-SNAP-SNARE fusion machinery (15), as discussed in the text. Some annexins, in particular annexin V and VI may also display a regulatory effect on the proposed functions of other annexin-family members (not shown).



the idea that annexins may function together with the NSF, SNAPs and SNARE-machinery.

As shown in Figures 7,8 and 10 there are many possible targets for annexins in vesicle trafficking and fusion. It is possible that only some members of the annexin family are able to act as fusogens, whereas others may even display inhibitory activity in membrane fusion, e.g. annexins V and VI (Gerke and Moss, 1997). From the evidence presented in this review, annexins II and VII are probably the most likely candidates for membrane fusogens. Other annexins may not be involved in the fusion process itself, but may be involved in transport to and aggregation of vesicles at fusion Sites, in regulation of the cytoskeleton at these sites, in organization of fusion membranes, regulation of other annexins and in Ca^{2+} signaling. Membrane fusogenicity appears to be restricted to a small sub-group of annexins, leaving other activities to be performed by other members of the family. However, some specificity of action may arise from the variant phospholipid and calcium-binding properties of annexins, which may cause differential localization to intracellular membranes as well as calcium requirement.

Annexins are expressed in protists and a wide range of multicellular organisms. However, they are not ubiquitous. No annexin gene nor any homologous structures are present in the genome of the yeast *Saccharomyces cerevesiae* (Braun *et al.*, 1998). From this, we can conclude that at least in this organism membrane fusion does not require annexins nor any related proteins. The evolutionary success of *Saccharomyces* points to a non-essential function of annexins in the processes where membranes fuse. In higher eukaryotes annexins may add a level of sophistication to membrane fusion processes, and certainly membrane fusion in mammals can be distinguished from equivalent events in *Saccharomyces cerevesiae*, for example with respect to Ca^{2+} requirement and kinetics. However, the molecular basis of these differences needs to be identified. Clearly, more work is needed to either prove or disprove the many roles that have been suggested for annexins in membrane fusion. In the future, gene knock-out models, in particular for annexins II and VII, will aid the evaluation of the roles of these proteins as membrane fusogens. Additionally, inducible expression systems, such as the tetracycline controlled expression system, which are already in preparation for some annexins, will no doubt provide crucial information about the exact role of annexins in membrane fusion.

ACKNOWLEDGMENTS. Work in the authors' laboratory is funded by the European Commission (contract BIO4CT960083), the British Heart Foundation and the Wellcome Trust.

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Chapter 4

The Full Complement of Yeast Ypt/Rab-GTPases and Their Involvement in Exo- and Endocytic Trafficking

Martin Götte, Thomas Lazar, Jin-San Yoo,
Dietrich Scheglmann, and Dieter Gallwitz*

1. INTRODUCTION

Eukaryotic cells are subdivided into membrane-enclosed compartments called organelles. Each organelle is endowed with a specific subset of lipids and cellular protein species according to its physiological specialisation. This fact requires means of sorting newly synthesized proteins with regard to their destination and a machinery to deliver them to their target compartment (Hendriks and Fuller, 1994). These membrane fusion and targeting processes have to be tightly regulated: For example, the surface area of the plasma membrane has to be kept constant by an equilibrium of exocytosis and endocytosis. Furthermore, nonspecific fusion processes between the membranes of different organelles have to be prevented to ensure cell integrity.

MARTIN GÖTTE, THOMAS LAZAR, JIN-SAN YOO, DIETRICH SCHEGLMANN, and DIETER GALLWITZ Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany. *Corresponding author.
Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

Different organelles have different modes of acquiring their individual protein and lipid constituents. Since the pioneering work of George Palade (1975) it is known that many proteins get to their final destination in membrane-derived transport vesicles. These specialized carrier units form at the donor compartment's membrane in a process referred to as budding. During this process, the vesicles are loaded with cargo molecules which can be sorted in a specific manner via interactions with cargo receptors (Kirchhausen *et al.*, 1997; Kuehn and Schekman, 1997). Coat protein complexes like COPI, COPII and clathrin, monomeric GTPases of the dynamin and ARF families as well as adaptor proteins take part in the budding and cargo selection processes which have been reviewed in detail elsewhere (Spang *et al.*, 1998; Springer and Schekman, 1998; Cosson and Letourneur 1997; Kirchhausen *et al.*, 1997; Kuehn and Schekman 1997; Nickel and Wieland, 1997; Rothman, 1994). It is thought that after budding, the transport vesicles shed their protein coat and travel to their target membranes along cytoskeletal structures (see previous reviews, and Hamm-Alvarez and Sheetz, 1998; Lippincott-Schwartz *et al.*, 1998a). Following synthesis at the ribosomes of the rough endoplasmic reticulum (ER), proteins enter the secretory pathway by translocation into the ER lumen. Provided it is folded correctly, the protein either stays in the ER if it possesses retention signals or continues its journey in transport vesicles moving to the Golgi apparatus (Klappa *et al.*, 1993; Rowling and Freedman, 1993). Glycosylation of some proteins starts in the ER and is further refined in different cisternae (*cis*, *medial*, *trans*) of the Golgi-apparatus. Further modifications like sulfations also occur in this organelle. While the Golgi-compartments of mammalian cells can be easily distinguished morphologically (Farquhar and Palade, 1981), different Golgi compartments in yeast are defined by specific glycosylating and processing enzymes (Franzusoff and Schekman, 1989). In a late Golgi compartment proteins are sorted according to their final destinations, i.e. the plasma membrane, endosomes or the lysosome. Targeting to endosomes and the vacuole partially overlaps with the endocytic pathway, which is characterized by internalization of molecules from the plasma membrane. Retrograde transport between exocytic- and endocytic compartments ensures that the qualitative and quantitative balance in the protein and lipid contents of the cell's organelles is maintained. Furthermore, they allow for recycling processes to occur (Emr and Malhotra, 1997; Rothman, 1994). Protein transport to peroxisomes, mitochondria and chloroplasts differs mechanistically from the vesicular delivery discussed here (Haucke and Schatz, 1997; Waterham and Cregg, 1997; Huhse and Kunau, 1995; Ellis and Reid, 1994; Robinson, 1994; van Veldhoven and Mannaerts, 1994). Apart from these transport processes, direct import pathways exist, which have been discussed elsewhere (Scott and Klionsky, 1997).

Formation and fusion of vesicles are regulated events. The vesicular transport routes described above as well as the basic machinery used to safeguard correct targeting and to accomplish fusion of intracellular membranes are well conserved among eukaryotes ranging from yeast to man (Ferro-Novick and Jahn, 1994; Bennett and Scheller, 1993). All eukaryotic cells investigated to date share molecular components with a role in membrane fusion which show strong homology to each other, emphasizing the importance of vesicular targeting. Baker's yeast *Saccharomyces cerevisiae* is a eukaryotic model organism easily amenable to genetic and biochemical analysis. Many components of the vesicular protein transport machinery were originally described in yeast. The entire information of the *S. cerevisiae* genome is now known and freely accessible (Mewes *et al.*, 1997). This allows us to determine the total complement of conserved protein families involved in vesicular protein transport in a prototypic eukaryotic cell. As far as current knowledge is able to tell, all membrane fusion in eukaryotic cells is governed by a class of monomeric Guanosine-triphosphate (GTP)-binding proteins, called Rab in mammals and Ypt in yeast (Lazar *et al.*, 1997; Novick and Zerial, 1997; Nuoffer and Balch, 1994). The GTPases are thought to act as molecular switches with an active, GTP-bound, and an inactive, GDP-bound conformation (Bourne, 1995; Bourne *et al.*, 1990). It is with this family of remarkably conserved regulatory proteins, called Ypts, that this review will concern itself. Each yeast cell contains eleven different types of Ypt proteins (Lazar *et al.*, 1997). Some of them serve functions which, even under optimal growth conditions, are absolutely required for the cell to remain alive, others are seemingly dispensable. Ypt proteins are grouped together as a family by virtue of common sequence motifs (Strom and Gallwitz, 1994; Gallwitz *et al.*, 1991). After a brief introduction to the Ras-superfamily of monomeric GTPases the reader will be familiarized with the structural features and posttranslational modifications common to all Ypt proteins. The functional cycling and the various states of Ypt proteins underlying their physiological action in membrane fusions as well as the proteins known to interact with the Ypt GTPases will be discussed. Following a description of the vesicle fusion machinery, current models of the mechanistic role of Ypt proteins in vesicular transport will be presented. An in-depth analysis of the function of every single member of the Ypt family leads to the question of how many Ypt proteins a eukaryotic cell needs to fulfil its physiological functions.

2. THE Ras-SUPERFAMILY

Ypt GTPases form a group within the superfamily of Ras-related proteins, named after its most prominent and best-studied member, the

proto-oncogene Ras (Wittinghofer, 1998; Bokoch and Der, 1993). Over 700 sequences from different species are known to belong to this superfamily. Using stringent criteria, Garcia-Ranea and Valencia (1998) have identified 29 members of this superfamily in *S. cerevisiae*, which comprises the Rho-, Arf-Bar- and Ran-families in addition to the Ras and Ypt proteins. The family members are characterized by common highly conserved GTP-binding regions and a C-terminal cysteine-containing motif that is post-translationally modified with a lipid moiety. Like Ras-proteins, Ypt GTPases made up from 200–230 amino acid residues. Furthermore, a similar spacing within the first 150–160 residues of the conserved guanine nucleotide binding regions G1–G5 is seen. The GTP-bound conformation represents the active molecule, which is inactivated upon GTP hydrolysis. A variety of effector proteins modulate the activity of the Ypt GTPases. These proteins as well as the structural features of the Ypt GTPases are discussed in the following sections.

In yeast, the Ras1- and Ras2-proteins play a regulatory role in the progression through the G1 phase of the cell cycle (Fasano, 1995a,b). The membrane-bound GTPases oscillate between a GDP-bound and a GTP-bound state, which are modulated by accessory factors such as GTPase activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). The GTP form activates adenylyl cyclase, resulting in the production of the second messenger cyclic adenosine monophosphate (cAMP), which in turn activates a cAMP dependent kinase, and ultimately a kinase cascade. Upon GTP hydrolysis, the Ras-GTPases undergo a conformational change which leads to their inactivation. This function as a regulatory switch is a recurring motif in all members of the Ras-superfamily (Kjeldgaard *et al.*, 1996). Apart from Ras and the Ypt GTPases, the other members play regulatory roles in processes as diverse as nuclear import (Ran), vesicle budding (Arf/Sar), cytoskeletal organisation and cell wall biosynthesis (Rho) (Hall, 1998; Lippincott-Schwartz *et al.*, 1998b; Melchior and Gerace, 1998; Bednarek *et al.*, 1996). Thus, the Ras superfamily is an excellent example for evolutionary diversification of a successful molecular invention, the GTPase switch.

3. THE Ypt PROTEIN: STRUCTURE

The Ypt proteins are grouped as a family within the Ras superfamily by virtue of sequence homology and characteristic sequence motifs. Sequencing of the *S. cerevisiae* genome has revealed the existence of eleven Ypt GTPases (Lazar *et al.*, 1997). Conserved regions indicative of family membership are shown in Figure 1. Due to the very high degree of sequence

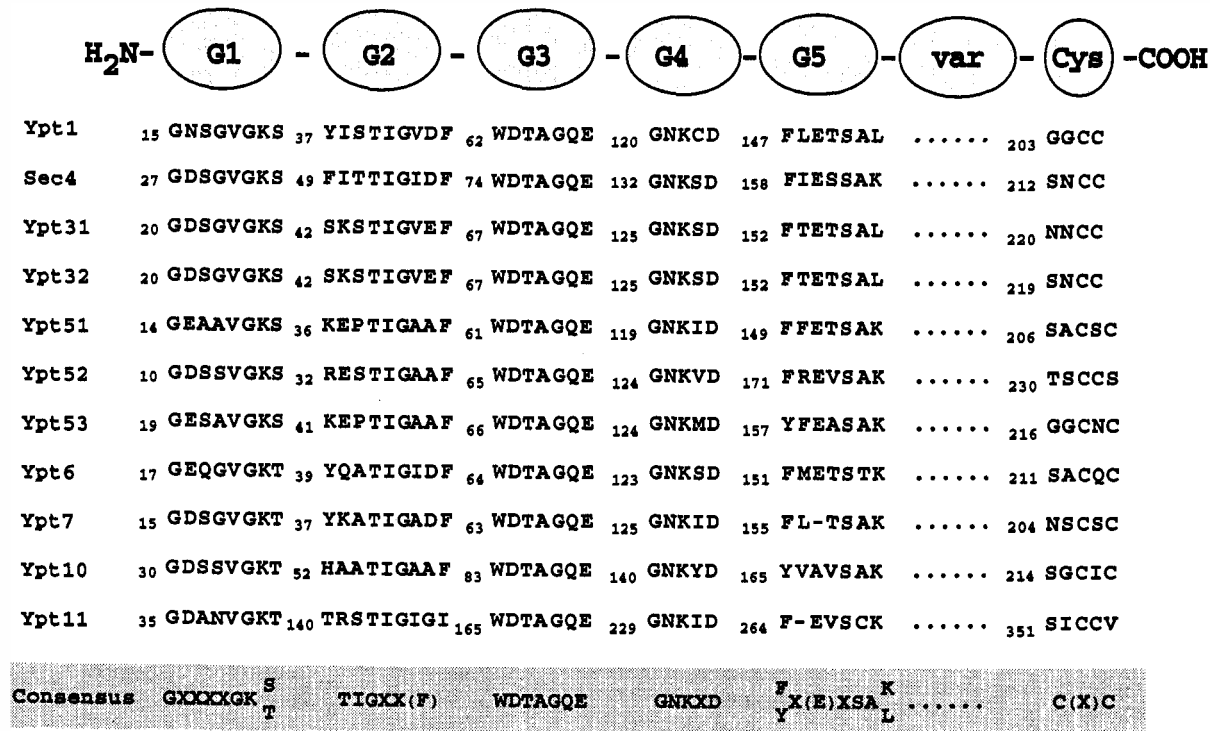


FIGURE 1. Conserved sequence motifs in Ypt GTPases. Members of the Ras superfamily of GTPases are characterized by structural motifs called G1 through G5. Conserved amino acid residues in these regions form the binding site and the reactive centre for the nucleoside phosphates and a Mg^{2+} -ion. Along with the hypervariable region, the effector domain G2 defines the functional specificity of the GTPase. Geranylgeranyl lipid chains are attached to the C-terminal cysteine residues necessary for the membrane insertion of the Ypt protein. Var = hypervariable region, Cys = conserved cysteine motif, Consensus = amino acid residues conserved between all Ypt GTPases.

homologies, all Ras-related proteins are assumed to basically adopt a very similar structure and thereby resemble the Ras protein whose spatial structure is known in great detail (Wittinghofer, 1998; Hilgenfeld, 1995). The G regions are known in Ras to fold up in such a way that they form the binding site and the reactive center for the nucleoside phosphates (Sprang and Coleman, 1998; Kjeldgaard *et al.*, 1996; Wittinghofer and Valencia, 1995). The G1-region forms a loop that is responsible for binding of the alpha and beta phosphate groups of GTP/GDP. The G3-region binds to the gamma-phosphate of GTP, whereas the G4-Region is responsible for binding the guanine ring. The conserved amino acid residues of the GS-region are required for stabilization of the G4-interactions. Of special interest is the so-called effector domain G2. It is the part of Ras-like GTP binding proteins which undergoes the most extensive conformational change upon hydrolytic cleavage of the gamma-anhydride bond of GTP (Kjeldgaard *et al.*, 1996; Wittinghofer and Nassar, 1996; Hilgenfeld, 1995). Ypt proteins are expected and predicted to show the same intramolecular re-arrangement after having lost the terminal phosphate of the bound nucleotide. The conserved threonine residue of the G2 region's TIG-motif binds a Mg^{2+} -ion which is essential for GTP hydrolysis. The effector domain is also thought to bind the target protein which receives the signal that the conformational change is relaying (Becker *et al.*, 1991). Along with the hypervariable C-terminal sequence, this region is important for the functional specificity of the Ypt GTPase.

Another common structural feature is the carboxyterminal cysteine-harboring sequence motif. On this act lipid transferases which post-translationally attach isoprenoid lipids. Ypt proteins are the substrate of geranylgeranyl transferase II (GGT II), a heterodimer of the proteins Bet4p and Bet2p (Witter and Poulter, 1996; Jiang *et al.*, 1993; Rossi *et al.*, 1991). In *bet2* mutants, both Ypt1p and Sec4p—and probably all other Ypt proteins—are unable to associate with their target membranes. In the case of mammalian Rab proteins, the Rab escort proteins (REP) present the GTPase to the prenylating enzyme. The structural homologue in yeast, Mrs6p, fulfils a similar function (Miaczynska *et al.*, 1997; Benito-Moreno *et al.*, 1994; Fujimura *et al.*, 1994; Waldherr *et al.*, 1993). It is by the isoprenoid lipid “tails” that the Ypt proteins are associated with their target membranes. Without this post-translational modification the proteins are completely disabled and cannot fulfill their physiological functions (Walworth *et al.*, 1989; Molenaar *et al.*, 1988). Ypt-GTPases of forward transport terminate in a double cysteine while the remaining relatives end with either an additional amino acid or possess a cysteine-X-cysteine motif. Whether this fact is consequential or coincidental is not known.

The mechanism by which interaction with the correct membrane is achieved is still poorly understood, but it appears that the hypervariable C-terminus is responsible for specific membrane interaction (Brennwald and Novick, 1993; Chavrier *et al.*, 1991). The recently described Yip1 protein is a candidate membrane receptor for Ypt GTPases (Yang *et al.*, 1998). GTP-GDP-exchange and hydrolysis as well as membrane attachment of the Ypt GTPases are tightly regulated through interactions with accessory proteins. These functionally important processes will be described in detail now.

4. THE GTPASE CYCLE

Ypt GTPases cycle between donor/membrane and target membranes, between a membrane-bound and a soluble, cytosolic form, and between a GTP- and a GDP bound state (see Figure 2). In the cell a fraction of the total amount of any Ypt protein is attached to membranes while the rest lies dormant in a cytosolic state, complexed by an interacting protein called guanine-nucleotide dissociation inhibitor (Gdi1p = Sec19p). Gdi1p is a protein which indiscriminately interacts with all types of Ypt proteins (Wu *et al.*, 1996). It is able to solubilize the inactive, GDP-bound Ypt protein from target membranes and guide it to the correct donor membrane (Maltese *et al.*, 1996; Garrett *et al.*, 1994; Araki *et al.*, 1990). A hypothetical

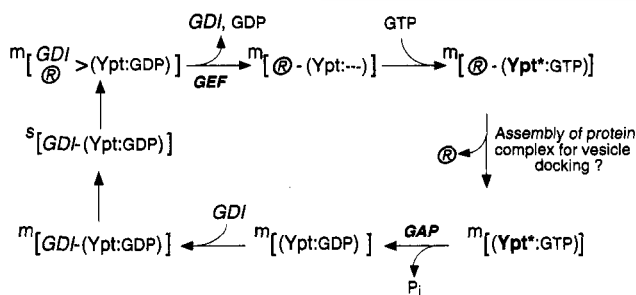


FIGURE 2. Model of the Ypt GTPase cycle (to be followed clockwise from middle left). The inactive GDP-bound form of the Ypt protein is kept in a soluble state by the GDI protein. Upon docking to a putative membrane receptor, GDI. The activated Ypt GTPase most likely acts in the assembly of a protein complex which facilitates membrane docking. Dissociation of the putative receptor-Ypt complex enables GAP-accelerated GTP hydrolysis. The cycle is completed by GDI extracting the inactive GDP-bound form of the Ypt GTPase from the membrane again. s = soluble, m = membrane-bound, Ypt* = activated form of the Ypt GTPase, R = membrane receptor for Ypt GTPase.

receptor has been postulated to be responsible for specific membrane binding (see Lazar *et al.*, 1997). After membrane association GDI dissociates from transport GTPases (Dirac-Swjestrup *et al.*, 1997; Soldati *et al.*, 1994; Ullrich *et al.*, 1994). Up to now, it is not known to which membrane (donor, vesicular or acceptor membrane) the Ypt/Gdi is transported for a newly insertion of the Ypt protein into the lipid phase. Except for allowing repeated use of the GTPase, the physiological meaning of the cycling between a membrane-attached and a cytosolic state is not known since it has been shown that this shuttling is not essential for proper Ypt protein functioning (Ossig *et al.*, 1995). It might serve to regulate the amount of membrane docking by fine-tuning the amount of Ypt protein available on the participating membranes.

In contrast to the dispensability of the oscillation between the membrane-bound and the free state of the molecule, interaction with a guanine-nucleotide exchange factor (GEF) is required (Jones *et al.*, 1995). Once the GDP-bound form of the transport GTPase associates with a membrane, GDP has to be exchanged for GTP in order to activate the GTPase (Macara *et al.*, 1996; Quilliam *et al.*, 1995). In the case of mammalian endosomal Rab GTPases, the action of a GDI-displacement factor (GDF) is needed to release GDI (Dirac-Swejestrup *et al.*, 1997). However, such a factor has not been identified in yeast. The soluble Sec2 protein has been described as a GEF for the Sec4p GTPase (Walch-Solimena *et al.*, 1997). Like *SEC4*, the *SEC2* gene is essential and its gene product seems to ensure that Sec4p activation is coupled to the polarized delivery of exocytic vesicles to the growing bud. A GEF-activity has recently been described for Ypt1p, the molecule involved, however, has not been identified yet (Jones *et al.*, 1998). The hydrophilic 17 kD protein Dss4p stimulates dissociation of both GDP and GTP from Sec4p. Dss4p is the yeast homologue of the mammalian exchange protein Mss4p, and its mutant form Dss4-1p was identified as a suppressor of multiple defects of the *sec4-8* mutation (Moya *et al.*, 1993). Recent results appear to exclude a role for Dss4p in the recruitment of a Sec4p/Gdi1p complex to membranes (Collins *et al.*, 1997). Since Ypt/Rab-GTPases display only a low intrinsic GTPase activity, GTP hydrolysis has to be accelerated. This is achieved in a regulated manner through the action of GTPase activating proteins (GAPs) (Scheffzek *et al.*, 1998; McCormick, 1998; Strom *et al.*, 1994). The structural properties of GAPs, GDI, and GEF have been reviewed recently (Scheffzek *et al.*, 1998; Sprang and Coleman, 1998; Geyer and Wittinghofer, 1997).

In recent years, many GAPs for Ras and Rho-proteins were isolated and their genes cloned (for reviews see Scheffzek *et al.*, 1998; Narumiya, 1996; Lamarche and Hall, 1994). Few GAPs that act monospecifically *in vitro* are known so far: a specific RapGAP for Rap1A and Rap2

(Rubinfeld *et al.*, 1992) or the two Ras-GAPs in *S. cerevisiae*, Ira1p and Ira2p, which are highly specific for yeast Ras1/2p and do not stimulate mammalian Ras (Parrini *et al.*, 1996; Tanaka *et al.*, 1991). Most of the several GAP-proteins seem to be multispecific, at least *in vitro* (Cullen *et al.*, 1995; Rey *et al.*, 1994; Boguski and McCormick, 1993; Bollag and McCormick, 1991; Garrett *et al.*, 1989). Little is known about GTPase activating proteins for the Ypt/Rab family of small GTPases. The first evidence for GAP-activity acting on Ypt1p was found in porcine liver (Tan *et al.*, 1991; Becker *et al.*, 1991) and specific GAP-activities for Ypt1p and Sec4p could be identified in pancreatic extracts (Jena *et al.*, 1992), suggesting crossreactivity between mammalian RabGAPs and Ypt proteins from baker's yeast. A great number of GTPase activating proteins for small GTPases have been identified by different screening methods, including genetic assays, a filter overlay assay (Manser *et al.*, 1992), or physical interaction of hybrid proteins (Park and Weinberg, 1995). The first gene for a Ypt-GAP, *GYP6* (GAP for Ypt6p) was cloned by screening extracts from yeast cells transformed with a DNA library for GAP-activity towards recombinant Ypt proteins (Strom *et al.*, 1993). Gyp6p *in vitro* acts preferentially on Ypt6p and only shows a 10% side activity towards Ypt7p. Subsequently, two other genes for Ypt-GAPs, *GYP7* and *GYP1*, were identified by the same method of expression cloning (Vollmer *et al.*, 1995; S. Albert, E. Will and D. Gallwitz, unpublished). *GYP1* was also cloned by Du *et al.* (1998) using the sequence information from the related *GYP7*. Gyp7p and Gyp1p act preferentially on Ypt7p and Ypt1p/Npt51p/Sec4p, respectively. At least in cell-free systems, the Ypt-GAPs are promiscuous with respect to their substrate GTPases. It is unclear whether this reflects the situation *in vivo* but the possibility of such a regulatory mechanism is shown in the case of human Ras where more than one GAP regulates p21 in the same cell (Bollag and McCormick, 1991). It was suggested that Rab3A-GAP, the only mammalian Rab-GAP known, is active not only on Rab3A, but also on other Rab3 isoforms (Fukui *et al.*, 1997; Brondyk *et al.*, 1995). It could be that there is a variety of GAPs with differing but overlapping activities like it is the case with Ras (Mollat *et al.*, 1994) and Rho GAPs (Ridley *et al.*, 1993). To abolish the GAP activity toward one small GTPase one therefore would have to simultaneously disrupt several GAP genes. Another GAP activity acting specifically on Ypt1p was partially purified and was found to be independent of Gyp1p and Gyp7p (Jones *et al.*, 1998), suggesting the existence of more than three GAPs for Ypt proteins.

The intrinsic GTPase activity of Rab7, the mammalian homologue of Ypt7p, shows a hydrolysis rate of $t_{1/2} = 120\text{min}$, whereas the Rab7-dependent transport step from early to late endosome takes about 1 hour (Simon *et al.*, 1996). Surprisingly, GTP hydrolysis does not seem to be

important for Ypt1p function in vesicular transport (Richardson *et al.*, 1998). Similar observations have been made for mammalian Rab proteins and have led to the proposition that these GTPases act as a timer of vesicle docking and/or fusion (Rybin *et al.*, 1996). The finding of Jones *et al.* (1998) that an apparent Ypt1p GEF activity fractionates with the acceptor fraction, and a novel GAP activity with the donor fraction, of an ER-Golgi transport assay shows that some issues concerning the cycling of Ypt GTPases are yet unresolved. A good working model, however, is the idea that the Ypt GTPase is kept in an inactive cytosolic form by the successive action of GAP and GDI to ensure that Ypt activation takes place only at the appropriate target membrane (see Novick and Zerial (1997) for discussion). Current models of the functioning of Ypt GTPases in the process of transport vesicle docking are described in the next section.

5. YPT GTPASES AND SNAREs: THE FUSION MACHINERY

Studies in *Saccharomyces cerevisiae* proved extremely useful in establishing a role for small Ras-related GTPases in vesicular protein transport. A massive accumulation of Golgi-derived transport vesicles that were incapable of docking with the plasma membrane were found in a mutant defective in the Ypt GTPase Sec4p (Salminen and Novick, 1987). Furthermore, a role for Ypt1p in docking of ER-derived vesicles with the *cis*-Golgi compartment was established both through *in vivo*—(Becker *et al.*, 1991) and *in vitro*—(Rexach and Schekman, 1991) experiments. The discovery and functional analysis of the Ypt7 protein revealed that Ypt-GTPases do not only act on the secretory, but also on the endocytic pathway (Wichmann *et al.*, 1992). The findings in yeast were corroborated by studies in mammalian cells where different Rab proteins localize to distinct organelles of the secretory and endocytic pathways (Novick and Zerial, 1997; Chavrier P. *et al.*, 1990a). In yeast further components of the vesicular fusion machinery were identified soon after a role of the Ypts in vesicular transport began to emerge. In a screen for suppressors of the lethality caused by loss of Ypt1p, Dascher *et al.* (1991) isolated genes encoding transmembrane proteins (SNAP receptors = SNAREs) which were later found to be parts of the core machinery of vesicle fusion. Furthermore, they found that a mutant form of a soluble negative regulator of fusion complex formation, Sly1-20p, was able to compensate for the absence Ypt1p. Another part of the vesicle fusion machinery, the gene encoding the t-SNARE (target membrane SNARE) Sec9p was isolated as a suppressor of a Sec4p effector domain mutation (Brennwald *et al.*, 1994). It soon turned out that the Ypt suppressors have neuronal homologues which act in regulated vesicle fusion at

the synapse, thus confirming the role of the Ypt-GTPases in membrane fusion events (Ferro-Novick and Jahn, 1994; Bennett and Scheller, 1993).

An extremely useful model for the description of the molecular events leading to membrane fusion is the SNARE hypothesis (Rothman, 1994; Söller *et al.*, 1993) which states that synaptobrevin-related transmembrane receptors (v-SNAREs) on the vesicle membrane specifically pair with transmembrane receptors of the syntaxin and SNAP-25 families (t-SNAREs) residing on the target membrane and ultimately drive the membrane fusion process. Due to their interaction with the N-ethyl-maleimid sensitive fusion protein (NSF for short) and soluble NSF attachment proteins (SNAPs) these molecules were named SNAREs for SNAP receptors. In a current view of this model, the membrane proteins resemble the core machinery of the vesicle fusion process, while other factors like Ypt- and Sec1-related proteins play a role the docking process and in determining the specificity of the membrane fusion process (Götte and Fischer von Mollard, 1998; Weber *et al.*, 1998; Hanson *et al.*, 1997; Hay and Scheller, 1997).

How do the Ypt GTPases mechanistically fit into the process of membrane fusion? Much has been learned from *in vitro* transport assays. Wickner and colleagues were able to determine the order of events leading to vacuole-vacuole docking and membrane fusion (Ungermann and Wickner, 1998; Ungermann *et al.*, 1998; Mayer and Wickner, 1997; Nichols *et al.*, 1997; Haas and Wickner, 1996; Mayer *et al.*, 1996; Haas *et al.*, 1995). In their test system, vacuoles are isolated from two different yeast strains. One strain is deficient in the gene encoding alkaline phosphatase (ALP) while the other strain does not contain the gene encoding the processing enzyme proteinase A (PrA = Pep4p). A proteolytic activation of the reporter enzyme alkaline phosphatase can only occur when the vacuoles of both strains fuse. This system does not only allow to measure vacuole fusion, but also vacuole-vacuole docking, since docked vacuoles are dilution-resistant. Donor and acceptor membranes can be incubated separately, and the protein composition of their membranes can be controlled by introducing mutations into the genome of the strains used for vacuole preparation. Prior to SNARE complex formation, existing protein complexes at the same membrane have to be disassembled. The complex consisting of the t-SNARE Vam3p, the SNAP-25 homologue Vam7p, the v-SNARE Nyv1p and the yeast homologue of alpha-SNAP, Sec17p is disintegrated by the ATPase Sec18p (yeast NSF). Sec17p is released into the cytosol prior to the docking step (Mayer *et al.*, 1996; Ungermann and Wickner, 1998). Sec18p action depends on the heterodimeric protein LMA1 which consists of thioredoxin and the proteinase inhibitor protein IB² (Xu *et al.*, 1997). LMA1 is thought to stabilize the Vam3p SNARE after separation from its complex

partners, thus preventing v-SNARE/t-SNARE re-pairing at the same membrane (Xu *et al.*, 1998). After this "priming" of the vacuoles, the actual docking is mediated by the Ras-like GTPase Ypt7p in an as yet ill-understood way (Mayer and Wickner, 1997). Ypt7p has to be present on both vacuoles to allow subsequent vacuole-vacuole fusion (Haas *et al.*, 1995). For optimal fusion rates, the v-SNARE Nyv1p and the t-SNARE Vam3p have to be present on opposite membranes (Nichols *et al.*, 1997). Attachment of the SNAP-25-related t-SNARE Vam7p to the vacuolar membrane is facilitated by its binding to Vam3p but not to Nyv1p (Ungermann and Wickner, 1998). Although Vam7p is not strictly required for the fusion process, it stabilizes the SNARE complexes on resting membranes. The high degree of thermodynamic stability achieved by the v-/t-SNARE pairing is thought to provide the driving force for the subsequent membrane fusion process, which seems to parallel viral fusion mechanisms in many respects (Götte and Fischer von Mollard, 1998; Weber *et al.*, 1998).

The order of events of membrane docking and fusion does not seem to be a peculiarity of homotypic membrane fusion, since similar results were found in an *in vitro* system measuring ER to Golgi transport, and in artificial liposome-based fusion systems (Cao *et al.*, 1998; Sato and Wickner, 1998; Weber *et al.*, 1998). Like in vacuole fusion, a Ypt GTPase (Ypt1p) acts at the docking step in the ER-to-Golgi transport assay (Cao *et al.*, 1998). It was shown that the docking reaction is sensitive to treatment with Gdi1p which removes Ypt1p from the membrane. As a consequence of the treatment, the amount of the membrane-associated docking protein Uso1p is reduced. After docking has occurred, the subsequent fusion step depends on the presence of V-SNAREs, t-SNAREs and Sly1p. These steps, however, are no longer sensitive to Gdi1p-treatment. Genetic and biochemical data suggest that, along with Ypt1p and Uso1p, the peripheral membrane protein Sec35p is also required during the docking step (VanRheenen *et al.*, 1998).

It appears that large multiprotein complexes determine the specificity of membrane fusion events at an early docking step which is often referred to as tethering (Götte and Fischer von Mollard, 1998; Lowe *et al.*, 1998). Several docking complexes have been isolated both in yeast and mammals: The TRAPP complex acts at the ER-Golgi transport step (Sacher *et al.*, 1998), the Exocyst complex acts in Golgi to plasma membrane transport (Kee *et al.*, 1998; TerBush *et al.*, 1996). Several gene products defective in the class I and class II *vam*-yeast mutants (Wada *et al.*, 1992) which display an aberrant vacuolar morphology, form putative docking complexes at the vacuolar membrane (Rieder and Emr, 1997; Nakamura *et al.*, 1997). It has been suggested that Ypt GTPases recruit docking factors from the cytosol (Schimmöller *et al.*, 1998) and the data from the *in vitro* fusion systems support this hypothesis.

Apart from a role in vesicle docking, other putative mechanistic roles for Ypt-GTPases in the docking and fusion process have been proposed: One assumed function is the removal of inhibitors of SNARE complex formation, namely proteins of the Sec1-family, from syntaxin-related t-SNAREs. This idea does not necessarily contradict an involvement of Ypt proteins in docking, since at least in the vacuolar Vam-docking complexes the Sec1 family member Vps33p is found (Rieder and Emr, 1998). However, in an ER-to-Golgi *in vitro* transport system Ypt1p action precedes the requirement for the Sec1-protein Sly1p (Cao *et al.*, 1998). Roles for Ypt1p both in v-SNARE activation (Lian *et al.*, 1994) and t-SNARE activation (Lupashin and Waters, 1997) have been proposed, but it has to be considered that, in contrast to *in vitro* systems, the assays used in these studies were not able to establish a temporal order of docking and fusion steps. Therefore, a role for Ypt proteins during the docking step preceding membrane fusion proper seems to be most likely according to current knowledge. It has been pointed out that both Ypt proteins and SNAREs act at specific routes along the secretory and endocytic pathways. In the following sections we will use the state of the art in the field in an attempt to relate each protein to a specific transport step.

6. THE Ypt FAMILY ONE BY ONE

We shall first discuss the Ypt proteins involved in centrifugal transport from the ER via the Golgi apparatus to the plasma membrane (Ypt1p, Ypt31p, Ypt32p, Sec4p), and we shall then describe the Ypt GTPases known to act in vacuolar and endocytic trafficking (Ypt51p, Ypt52p, Ypt53p, Ypt7p). After a discussion of the role of the Golgi-associated Ypt6 protein, two Ypt proteins with yet undefined roles will be introduced (Ypt10p, Ypt11p).

6.1. Ypt1p

The history of exploration of the role of GTP-binding proteins in baker's yeast began in the early eighties with the discovery of the first member of what should ultimately become an extended kinship of genes and their corresponding proteins (Gallwitz *et al.*, 1983). Destruction or removal of the *YPT1* gene from the cell proved to be fatal to the organism (Segev and Botstein, 1987; Schmitt *et al.*, 1986). Functional *YPT1* homologues have been isolated from species as diverse as mouse (Haubruck *et al.*, 1989; Haubruck *et al.*, 1987), *Volvox carteri* (Fabry *et al.*, 1995, Fabry *et al.*, 1993), Soybean (Kim *et al.*, 1996) and *Phytophthora infestans* (Chen

and Roxby, 1996), to name but a few. Consecutive probing into its physiological role revealed that *S. cerevisiae* Ypt1p acts in vesicular protein transport from the ER to the early Golgi compartment. Yeast cells depleted of Ypt1p by shutting down expression from an inducible promotor, as well as conditional-lethal *ypt1* mutants at the nonpermissive temperature, are characterized by phenotypic alterations such as accumulation of ER membranes, 50nm vesicles and ER-core glycosylated proteins (Jedd *et al.*, 1995; Becker *et al.*, 1991; Bacon *et al.*, 1989; Schmitt *et al.*, 1988; Segev *et al.*, 1988). *In vitro* protein transport studies have shown that Ypt1p is required for ER-to-Golgi transport (Segev, 1991; Rexach and Schekman, 1991; Baker *et al.*, 1990), most likely for the docking of ER-derived vesicles to the *cis*-Golgi, the subcellular location at which Ypt1p is predominantly found (Segev *et al.*, 1988).

Studies on the *ypt1* alanine to aspartate mutation A136D, which leads to a very rapid and tight blockade of transport at the nonpermissive temperature, suggest an additional role in transport from the *cis*- to the *medial*-Golgi compartments. From these experimental results it has been concluded that the Ypt1 protein is essential for the first two steps of the yeast secretory pathway (Jedd *et al.*, 1995). Two additional mutations, Ypt1 asparagine 121 to isoleucine, (N121I) and Ypt1 aspartate 124 to asparagine (D124N) confer growth inhibition and a blockade of protein secretion upon the cell when expressed *in vivo*. The purified mutant proteins are defective in nucleotide binding (Jones *et al.*, 1995; Schmitt *et al.*, 1986). In the case of the (D124N) mutation the nucleobase-specificity is changed from guanine to xanthine. Therefore the block in the (D124N) mutant can be alleviated by providing the cell with XTP. Both mutations decrease or utterly block the activity of a partially purified guanine exchange factor (GEF). Interestingly, the dominant negative effect of the Ypt1 (N121I) mutation (Schmitt *et al.*, 1986) can be overcome by expression of the Ypt31 (N121I) mutant protein, but not by expression of analogous mutant versions of Ypt6, Ypt7 or Sec4 proteins, suggesting overlapping functions of the transport GTPases Ypt1p and Ypt31/32p (Yoo *et al.*, 1999). This idea is further supported by the identification of a common interacting partner for the Ypt1p and Ypt31p GTPases, Yip1p, in a two-hybrid-screen for interacting partners of Ypt1p (Yang *et al.*, 1998). Like its presumed partner, the Golgi-associated membrane protein Yip1p is essential for cell viability. Depletion of Yip1p results in a severe underglycosylation of secreted invertase and an accumulation of the ER-glycosylated forms of the vacuolar hydrolases carboxypeptidase Y (CPY) and alkaline phosphatase (ALP). At the ultrastructural level, an accumulation of ER membranes could be observed in Yip1p depleted cells. Surprisingly, Yip1p in the two hybrid system also interacts with Ypt31p, supporting the notion that the areas of influence of Ypt1p

and Ypt31p overlap to a limited extent at least. Therefore, Yip1p appears to act in ER-Golgi and/or early Golgi membrane transport, possibly as a membrane receptor for Ypt1pNpt31p.

Employing a *GAL10-YPT1* promotor-gene fusion, four genes, including a mutated allele of one of the suppressors capable of suppressing the loss of Ypt1 function (*SLY*), were isolated and characterized (Dascher *et al.*, 1991; Ossig *et al.*, 1991). *SLY2* and *SLY12* are identical to *SEC22* and *BET1*, respectively, two genes that are involved in ER to Golgi transport (Newman and Ferro-Novick, 1987; Novick *et al.*, 1980). Interestingly, the v-SNAREs Sly2 and Sly12 proteins are components of the membrane fusion-complex including the *cis*-Golgi membrane receptor Sed5p (Søgaard *et al.*, 1994). Sly1p, as well as Sly1–20p, binds Sed5p with high affinity (Grabowski and Gallwitz, 1997). The experiments failed to pinpoint Ypt1p as a constituent of the membrane-fusion complex, which is not surprising since Ypt1p seems to be involved in the docking rather than the vesicle fusion event. The mechanistic role of Ypt1p in docking of ER-derived vesicles to membranes of the *cis*-Golgi compartment has been discussed in section 5.

6.2. Ypt31pNpt32p

Assuming there could be a homologue of the mammalian Rab11 and *Schizosaccharomyces pombe* Ypt3 proteins, the genomic DNA of baker's yeast was probed by PCR and by hybridizing it to a fission yeast *YPT3* probe. Under conditions of low stringency, a pair of *YPT* genes, named *YPT31* and *YPT32* by virtue of their relatedness to the fission yeast's gene, were identified (Benli *et al.*, 1996). *YPT31* was independently discovered and provisionally given the name *YPT8* (Lai *et al.*, 1994). Ypt31p and Ypt32p display a striking 80% sequence identity: both of them exhibit more than 60% sequence identity with *Schizosaccharomyces pombe* Ypt3p and the canine Rab11a and Rab11b proteins. Furthermore, they share an identical effector region (SKSTIGVEF) with both of these proteins. While single disruptions of either *YPT31* or *YPT32* are phenotypically neutral, disrupting both is fatal. For functional studies *ypt32* deletion strains were depleted of Ypt31p using either a short-lived ubiquitinated form of Ypt31p or a galactose-inducible construct (Benli *et al.*, 1996). Depletion of Ypt31p, which is considered to be 5–10x more abundant than Ypt32p (Jedd *et al.*, 1997), led to an accumulation of Golgi-membranes and structures similar to so-called "Berkeley-bodies" (Novick *et al.*, 1980). Highly glycosylated invertase accumulated intracellularly one hour after Ypt31p depletion while at later points in time underglycosylated invertase could be detected. Fourteen hours after shut-off of Ypt3p's synthesis, maturation defects of vacuolar enzymes were seen. The delayed maturation of proteinase A (PrA

= Pep4p), CPY and ALP is considered a secondary effect of the blockade of intra-Golgi transport. Identical observations were made using the heat-sensitive allele *ypt31-1* which harbours a K127N amino acid exchange (Benli *et al.*, 1996), and in the *ypt32-1* allele (Jedd *et al.*, 1997). Interestingly, these protein transport defects are similar to the phenotypes of two known *S. cerevisiae* Golgi apparatus mutants, *sec7* and *sec14* (Franzusoff and Schekman, 1989). Studies on the localization of Ypt31p support the idea of a function in a Golgi compartment: A large part of the total cellular Ypt31p is found in Golgi-enriched membrane fractions when subcellular fractionations are carried out. Furthermore, the punctate Golgi-like staining obtained by immuno-fluorescence microscopy suggests binding to Golgi apparatus membranes. This can be interpreted in such a way as to implicate Ypt31p in intra-Golgi transport or in vesicle formation at the distal Golgi sub-compartment, or the trans-Golgi network (TGN) (Jedd *et al.*, 1997; Benli *et al.*, 1996). As has been pointed out previously (Lazar *et al.*, 1997; Benli *et al.*, 1996), it is well possible that the Ypt31/32 GTPases have a function in retrograde rather than in anterograde Golgi transport. A systematic analysis of the genetic interactions between *YPT1*, *YPT31/32*, *ARF1* and *SEC4* supports the view that Ypt31p/Npt32p have a central Golgi-associated function (Yoo *et al.*, 1999). As mentioned above, the functions of Ypt1p and the Ypt31/32 GTPases seem to overlap partially. This idea is supported by the observation that both GTPases bind to the Yip1-protein (Yang *et al.*, 1998), that *YPT31* and *YPT32* are high copy number suppressors of a mutant Ypt1(D124N)p (Jedd *et al.*, 1997) and that mutant alleles of *YPT31* and *YPT32* are able to suppress the (N1211) mutation in the Ypt1 protein (Yoo *et al.*, 1999). Overlapping functions at the level of the Golgi-apparatus are not necessarily surprising, since this organelle can be divided into functional subunits which are connected both by retrograde and anterograde trafficking events (see introduction). In fact, besides four Ypt proteins (Ypt1p, Ypt31p, Ypt32p and Ypt6p), a large number of SNARE-proteins with a presumed transport function at the Golgi-level have been identified (Pelham, 1998). Thus, the complexity of the Golgi apparatus seems to require more than one set of vesicular trafficking proteins for proper function.

6.3. Sec4p

Chromosome VI is home to the *SEC4* gene which, in spite of its deviant name, cannot deny its belonging to the *YPT* kinship. The *SEC4* gene encodes a typical GTP-binding protein of 215 amino acids which is 47.5% identical to Ypt1p (Salminen and Novick, 1987). Homologues of Sec4p have not only been described in mammals (Chavrier *et al.*, 1990b) but also in

organisms as diverse as *Trypanosoma brucei*, *Candida albicans* and fission yeast (Clement *et al.*, 1998; Field and Field, 1997; Haubruck *et al.*, 1990). In the case of Sec4p, most of the previously mentioned accessory proteins that modify the localisation and the GTP/GDP binding state of Ypt GTPases are identified. These include Gdi1p (Sec19p) (Garret *et al.*, 1993), the GAP Gyp1p (Du *et al.*, 1998; S. Albert, E. Will and D. Gallwitz, unpublished), the GEF Sec2p (Walch-Solimena *et al.*, 1997), Dss4p (Moya *et al.*, 1993) and geranylgeranyl transferase II, which has been shown to modify the C-terminal cysteines of both Sec4p and Ypt1p (Rossi *et al.*, 1991).

The essential Sec4 protein is involved in the very last step of the secretory pathway, the fusion of vesicles stemming from the latest Golgi subcompartment or the *trans*-Golgi network (TGN) with the plasma membrane (Salminen and Novick, 1987; Field and Schekman, 1980). The temperature-sensitive (ts) allele *sec4-8* carries a glycine to aspartate amino acid exchange at position 147 (G147D) and was used in several studies on Sec4p function. The mutant displays a blockade of the transport of the enzyme invertase and accumulates secretory vesicles 100nm large, containing fully processed cell surface proteins, within 5 minutes upon shift to the nonpermissive temperature (Salminen and Novick, 1987). While Sec4p primarily localizes to the plasma membrane in wild-type cells, it is mainly bound to the accumulated vesicles in *sec4* ts-mutants (Goud *et al.*, 1988). As is the case with Ypt1p, a permanently membrane-anchored form of Sec4p still permits unimpaired vesicular transport to take place (Ossig *et al.*, 1995), suggesting that detachment of the GTPases from the membrane after GTP hydrolysis is not obligatory for transport vesicle docking or fusion with an acceptor membrane. However, it appears that the interaction of the Sec4p-GEF Sec2p couples GTPase activation and the polarized delivery of exocytic vesicles to the forming bud (Walch-Solimena *et al.*, 1997). This is in accordance with observations describing diploid-specific bud-site selection defects of *sec4-8* mutants and a disrupted actin cytoskeleton in these cells (Finger and Novick, 1997; Mulholland *et al.*, 1997). Reorganisation of the actin cytoskeleton is an important event in bud formation and is mediated by members of another Ras-subfamily, the Rho proteins (Hall, 1997). Sec4p colocalizes with Sec8p and Sec3p, which has been described as a spatial landmark that might establish the vesicle fusion site (Finger *et al.*, 1998; Finger and Novick, 1998; Finger and Novick, 1997). Several genetic interactions between *SEC4* and mutant forms of other late-acting *SEC* genes (*SEC2*, *SEC8*, *SEC15*, and to a smaller extent *SEC1*, *SEC5*, *SEC10*, and *SEC19*) have been described (Salminen and Novick, 1987). Since some of the proteins encoded by these genes have been found in multiprotein complexes, Sec4p seems to be a regulator of complex formation, possibly at the docking step (TerBush and Novick, 1995; Bowser *et al.*, 1992; Bowser and

Novick, 1991). Interestingly, the *SEC9* gene has been identified as a suppressor of Sec4p effector domain mutations (Brennwald *et al.*, 1994). Displaying significant structural similarity to the neuronal t-SNARE SNAP-25, the Sec9 protein is found in association with the plasma membrane t-SNAREs Sso1p and Sso2p (Rossi *et al.*, 1997; Aalto *et al.*, 1993). Sec4p function can therefore be linked to the same transport step that is governed by Sec1p, Sec9p and the Sso/Snc-SNARE complex.

6.4. Ypt51p/Ypt52p/Ypt53p

Prior to a discussion of the Ypt proteins 5–7, we will give a brief introduction of protein transport to the yeast vacuole, which will be helpful in understanding the roles played by these GTPases. Upon glancing at individual yeast cells under a microscope the most conspicuous organelle is the vacuole. It occupies approximately one quarter to one third of the cell's total volume. The acidic lumen of this organelle is highly enriched in hydrolytic activity. The vacuole is the functional homologue of the mammalian lysosome and the plant vacuole (Conibear and Stevens, 1998; Robinson and Hinz, 1997; van den Hazel *et al.*, 1996). It serves as a deposit of useful macro- and micronutrients such as recycled amino acids and inorganic ions (Bode *et al.*, 1995; Jaquemin-Faure *et al.*, 1994). The vacuole is also the primary site of degradation and recycling of nutrients and is therefore a regulatory instance of major importance for maintaining and securing the cell's homeostasis (Bone *et al.*, 1998; Shirahama *et al.*, 1996; Jaquemin-Faure *et al.*, 1994). Apart from nonvesicular trafficking (Scott and Klionsky, 1997), various vesicular pathways converge upon the vacuole: coated vesicles of the secretory pathway, the endocytic pathway, and possibly a new pathway of delivering cytosolic constituents (Conibear and Stevens, 1998; Huang and Chiang, 1997). Studies on the endocytosis of the mating pheromone alpha-factor and its receptor have revealed the existence of at least two kinetically and morphologically discernible compartments resembling early and late endosomes in mammalian cells (Prescianotto-Baschong and Riezman, 1998; Hicke *et al.*, 1993; Singer-Krüger *et al.*, 1993). The so-called prevacuolar compartment seems to correspond to the late endosome. This organelle participates in recycling processes involving the most distal Golgi-compartment and resembles one of two major anterograde transport routes to the vacuole. Over 40 *VPS*-genes have been described (Conibear and Stevens, 1998; Robinson *et al.*, 1988). Yeast cells mutated in these genes are defective in transport of hydrolases to the vacuole and in many cases show an aberrant vacuolar morphology. Given the diversity and multitude of pathways leading to the vacuole, it is not surprising that a considerable number of genes are involved in the

differential regulation of the various routes and gates. Among these found are four out of the eleven *YPT* genes. They will be discussed in greater detail below.

Ypt51p, Ypt52p and Ypt53p have been grouped together because these isoforms share a high degree of sequence similarity and an identical effector region with mammalian Rab5 (Singer-Krüger *et al.*, 1994). While two members (*YPT51* (= *VPS21*) and *YPT53*) were identified in a low-stringency PCR-screen, the sequence of *YPT52* was uncovered by the total sequencing of chromosome XI. The high degree of sequence similarity to Rab5 suggested a similar function for the Ypt5 proteins. Heterologous expression of Ypt51p in mammalian cells indeed resulted in a stimulation of endocytosis and association of the GTPase with Rab5-positive endosomes. A constitutively activated mutant form, Ypt51(Q66L)p leads to the same biochemical and morphological changes as the corresponding mutation in the Rab5 protein (Singer-Krüger *et al.*, 1995). Analysis of yeast mutants deleted in one or several of the YPTS genes led to the discovery that all the three genes are likely to play roles in delivering proteins to the vacuole, either from the cell surface or from outside the cell by endocytosis, or from the Golgi apparatus (Horazdovsky *et al.*, 1994; Singer-Krüger *et al.*, 1994). Among the *YPT5* group, the strongest phenotype is seen upon deletion of the *YPT51* gene. *ypt51* mutants were also identified as *vps21* in a screen for yeast cells that secrete the precursor form of the vacuolar protease carboxypeptidase Y. In addition to the protein sorting defect, the *ypt51* phenotype shows other alterations: the acidification of the vacuoles is disturbed and the vacuole becomes enlarged upon deletion of the *YPT51* gene. The uptake of receptor-bound alpha-factor peptide is inhibited, as is delivery to the vacuole of the dye lucifer yellow CH during internalization by fluid-phase endocytosis. Genetic interactions with other members of the *VPS* family link *YPT51* and its protein product, Ypt51p, to the fusion machinery at a prevacuolar, endosomal compartment (Burd *et al.*, 1997; Horazdovsky *et al.*, 1995). Burd *et al.* (1997) have described genetic and physical interactions of the t-SNARE Pepl2p and its gene at the prevacuolar transport step. Apart from Sec18p (NSF) and the Sec1-like protein Vps45p, the Vac1 protein was identified. In *vac1*-deletion strains, both Pepl2p and Ypt51p show a subcellular fractionation pattern different from wildtype cells. Along with the similar deletion phenotypes of *ypt51* and *pep12* cells, this argues for a role at a neighbouring or the same vesicular transport step. Genetic interactions were also established between *YPT51/PS21* and *VPS8*. Membrane association of the RING finger protein Vps8p, which is needed for sorting of soluble vacuolar hydrolases, depends on Ypt51p function (Horazdovsky *et al.*, 1996). The connection of Ypt51p to the endocytic and vacuolar protein sorting pathways was recently

confirmed in a screen for synthetic lethality with a *ypt51* disruption. In addition to genes linked to actin cytoskeleton function, *VPS41* and genes of the endocytic pathway were isolated (Singer-Krüger and Ferro-Novick, 1997).

None of the *YPT5* genes were found to be an absolute need to the yeast cell, and even the triple mutant *ypt51/ypt52/ypt53* was viable under laboratory conditions (Singer-Krüger *et al.*, 1994). While the single deletions of *YPT52* or *YPT53* did not result in any obvious phenotype, double or triple deletions in combination with *ypt51* led to an aggravation of the defects seen in *ypt51* mutants. Interestingly, in a genome-wide expression study, the members of the *YPT5* group in *S. cerevisiae* were the only *YPT*-genes that showed a significant difference in mRNA levels after the so-called diauxic shift from fermentation to respiration (DeRisi *et al.*, 1997). A similar time-course of differential mRNA-expression was seen among the *YPT5* genes, suggesting that they might indeed act in concert. Yet, it remains to be shown whether Ypt52p and/or Ypt53p fulfil duties different from Ypt51p.

6.5. Ypt7p

Like other members of the *YPT*-family, *YPT7* was cloned employing a PCR strategy based on degenerate oligonucleotides and low stringency hybridization (Wichmann *et al.*, 1992). The *YPT7* gene localizes to chromosome XIII and encodes a Ras-type GTP-binding protein of 208 amino acids. Disruption of the *YPT7* gene leads to a *vps* mutant phenotype whose most conspicuous manifestation is a fragmented vacuole. The resultant biochemical phenotype of the *ypt7* mutation is an inhibition of the maturation of soluble vacuolar enzymes as exemplified by the investigation of the maturation of CPY and subsequent secretion of the Golgi-proform (p2CPY) of this enzyme (Wichmann *et al.*, 1992). Processing of the vacuolar enzyme alkaline phosphatase is also adversely affected in *ypt7* cells (Wichmann *et al.*, 1992). In addition to the missorting of vacuolar proteins, *ypt7* cells exhibit a blockage in the (vacuolar) degradation of internalized alpha-factor, a yeast mating pheromone peptide. These combined effects suggested a role for the Ypt7 protein at a late step of the endocytic route as well as a role in the trafficking of vacuolar proteins traversing the biosynthetic pathway en route to their final destination. Later studies were able to show the vacuolar membrane to be the prime place of residence of the Ypt7 protein (Haas *et al.*, 1995). Due to the limited resolution at the light microscopy level of the applied immunofluorescence approach, additional localization of the Ypt7 protein to other cellular structures such as vesicles cannot be ruled out with certainty. As has been described in detail in section

5, Ypt7p plays an important role in the docking step of homotypic vacuole fusion. Antibodies to Ypt7p inhibit the docking reaction in the *in vitro* vacuole fusion assay (Haas *et al.*, 1995). The inability of the vacuoles to fuse can be seen as the underlying cause for the vacuole fragmentation seen in *ypt7* mutant cells.

Much has been learned by studying the structure and action of Ras proteins from animal (including human) cells. These investigations have provided insights into which amino acids play which roles during the on/off oscillation of the monomeric G-proteins. Most of these important amino acids are involved in the binding of the nucleoside phosphate(s) and/or its chemical conversion while others are known or suspected to actively contribute to interactions with other proteins (see sections 2 and 3). Many of the amino acid exchanges which effect proper functioning of Ras proteins (Wittinghofer and Valencia, 1995) have been made for Ypt7p, too. Surprisingly, many of them had no detectable detrimental effect on the protein's functional integrity. However, a yeast strain carrying the mutant Ypt7(T22N)p which preferentially binds GDP, displays a phenotype similar to the *ypt7* null mutant (Wada *et al.*, 1996). A perturbation of lysosomal transport has also been observed in an analogous mutant of *Dictyostelium discoideum* (Buczynski *et al.*, 1997). In wild-type yeast cells, the time during which Ypt7p stays in the active, GTP-bound form appears to be controlled through interactions with the GAP Gyp7p (Vollmer *et al.*, 1995).

The findings from *in vivo* studies of *ypt7* mutants and the vacuole fusion *in vitro* system suggest at least two functions for Ypt7p: one in trafficking between a late endosomal compartment and the vacuole, and one in homotypic vacuole fusion. A role for the fission yeast homologue of Ypt7p in vacuole fusion was demonstrated *in vivo* recently (Bone *et al.*, 1998). In *Schizosaccharomyces pombe*, the vacuolar fission/fusion process depends on the Ypt7 and Ypt4GTPases (the latter not being present in baker's yeast) and is a physiological response to adjusting the intracellular osmotic pressure to changing environmental conditions (Bone *et al.*, 1998).

6.6. Ypt6p

The cells of multicellular organisms contain a much greater variety of Ypt proteins than single-celled microbes such as yeast. Not surprisingly, not all of them have homologues in yeast. One of them, the mammalian Rab6, does have homologues with an identical effector region in both baker's and fission yeast, though: The *Schizosaccharomyces pombe* Ryh1p with 69% sequence identity (Hengst *et al.*, 1990), and the *S. cerevisiae* Ypt6p with 75% identity (Hengst *et al.*, 1995). YPT6 was identified and cloned using

the *S. pombe ryhl* gene as a probe (Hengst *et al.*, 1995). Ypt6p is dispensible for *S. cerevisiae* but the cells become heat-sensitive upon loss of the *YPT6* gene (Tsukada and Gallwitz, 1996; Li and Warner, 1996; Hengst *et al.*, 1995). Both the *Arabidopsis thaliana* and the *H. sapiens* Rab6 proteins can complement the *ypt6* mutation in baker's yeast (Bednarek *et al.*, 1994; Beranger *et al.*, 1994), suggesting functional conservation of this protein. The intrinsic GTPase activity of Ypt6p can be specifically accelerated more than a hundred-fold by the GTPase activating protein (GAP) termed Gyp6p (Strom *et al.*, 1993). This cytosolic 54kD protein to a limited extent also enhances the GTP-hydrolyzing activity of Ypt7p but is not reactive towards Ypt1p or Ypt31p. Disruption of the gene is phenotypically neutral—meaning the cell's phenotype is not detectably altered by the manipulation; double disruptions of *GYP6* and *YPT6* do not show an aggravation of the mutant phenotype of *ypt6* strains (Strom *et al.*, 1995).

Both morphological and biochemical alterations can be observed in cells lacking Ypt6p: *ypt6* null mutants contain several small vacuoles instead of a single large vacuole. At the ultrastructural level, accumulation of 50nm vesicles and the appearance of membrane-bounded structures of a diameter of 150–300nm can be observed (Tsukada and Gallwitz, 1996). Indirect immunofluorescence light microscopy using affinity-purified, polyclonal anti-Ypt6p-antibodies resulted in a punctate staining reminiscent of Golgi-localized proteins (Hengst *et al.*, 1995). Interestingly, part of the Golgi-localized Kex2 protease is missorted to the vacuole in *ypt6* null mutants (Tsukada and Gallwitz, 1996). In addition to the mature form, a highly glycosylated precursor form of the mating pheromone alpha-factor is secreted in these strains. Furthermore, a partial missorting of the vacuolar hydrolase CPY can be observed. Taken together, these phenotypes of *ypt6* null mutants and the putative Golgi localization suggest a not precisely understood role for Ypt6p in a late Golgi transport step, possibly in the retrieval of Golgi proteins at the TGN-level. An assessment of Ypt6p function has been complicated by studies that relied on a temperature sensitive strain (*ypt6 ts*) expressing nonfunctional Ypt6p (stop codon at position 64) (Li and Warner, 1998; Li and Warner, 1996). The authors initially suggested a role for Ypt6p at an early step of the secretory pathway (Li and Warner, 1996), based on the observation that this *ypt6 ts* strain at restrictive conditions exhibits a partial defect in the secretion of invertase and an accumulation of the ER-modified precursor form of CPY. In addition, a defect in ribosome biosynthesis was seen. It has, however, to be noted that different genetic backgrounds of the *ypt6* strains seems to influence this phenotype. Furthermore, dominant negative effects of the partial *YPT6* deletion in the *ts*-strain cannot be ruled out. Li and Warner (1998) also showed that high expression of *YPT1* can suppress both the *ypt6 ts* strain and a *ypt6* deletion

strain in the same (*SSD1-d*) genetic background. Furthermore, the single-copy suppressor allele for loss of *YPT1*, *SLY1-20* (Dascher *et al.*, 1991) was able to suppress the growth defect of the *ypt6 ts* mutant. These observations were seen by the authors as further support for a role of Ypt6p at an early transport step from the *cis*- to the *medial* Golgi-apparatus. Interestingly, in *ts*-suppressor-screens using the *ypt6 ts*-mutant and the *ypt6* deletion strain with different genetic backgrounds, identical suppressor genes were isolated (Li and Warner, 1996; Tsukada and Gallwitz, 1996). While the construction of a yeast strain carrying a conditional allele of *YPT6* seems to be the best solution in addressing Ypt6p function, the analysis of the suppressors of the heat-sensitivity of *ypt6* null mutants (*SYS* genes) (Tsukada *et al.*, 1999; Tsukada and Gallwitz, 1996) might also contribute to understand the function of this GTPase. Apart from remedying the growth defect, overexpression of the *SYS* genes results in an alleviation of the CPY missorting of *ypt6* null strains. Double disruptions of one of the *SYS* genes and *YPT6* led to an aggravation of the phenotypes seen in *ypt6* deletion mutants. Studies of several Sys proteins are in line with the assumption that the Ypt6 GTPase and these proteins function in transport between the endosomal compartment and the most distal Golgi compartment (Tsukada *et al.*, 1999; Tsukada and Gallwitz, 1996).

6.7. Ypt10p

In contrast to the GTPases described above, it is much harder to clearly attribute a role to the Ypt proteins discussed in this and the following paragraphs to a specific vesicular transport step. The sequences of the *YPT10* and *YPT11* genes were revealed upon elucidation of yeast's entire genome (Maurer *et al.*, 1995; Doignon *et al.*, 1993). The proteins encoded by these genes had previously not been identified in functional screens. The existence of the Ypt10 protein has been predicted by taking a close-up look at the base sequence of the right arm of chromosome II. Aligning its deduced amino acid sequence to the ones of known Ras-like proteins assigned it to the Ypt subgroup. The authors of the initial discovery gave a mammal's Rab5 protein as closest match (Doignon *et al.*, 1993). None of the experiments carried out so far was revealing: destruction of the gene does not render the cell disabled in any observable manner (E. Will and D. Gallwitz, unpublished). A role for Ypt10p on the secretory or endocytic pathways has not been established, yet. It has, however, to be considered that the deletion of other genes with an established role in vesicular transport results in no visible, or rather subtle, phenotypes. The genes encoding the v-SNARE Nyvlp (Nichols *et al.*, 1997) and the t-SNARE Tlg2p (Abeliovich *et al.*, 1998; Holthuis *et al.*, 1998a; Sèron *et al.*, 1998) are prominent examples.

Therefore, unconventional approaches and novel techniques have to be employed for functional analysis of the *YPT10* gene product. The protein sequence of Ypt10p contains a so-called PEST motif rendering it prone to rapid degradation. Whether this is meaningful in a physiological sense is unclear. However, experiments performed in the authors laboratory suggest that Ypt10p might indeed be an unstable protein (E. Will and D. Gallwitz, unpublished).

6.8. Ypt11p

The genome sequencing project allowed to identify one more member of the *YPT* gene family, named *YPT11* (Lazar *et al.*, 1997). With 355 amino acids its translation product has a relative mass of some 41 kD, making it the largest and the odd member of the Ypt family. This protein bears features justifying its family membership: at its very carboxy-terminus we find a Cys-Cys-Val tripeptide which might be a target for a lipid transfer protein of the GGT I or farnesyltransferase type. The most conspicuous feature of this non-typical member of the Ypt family is a long insertion of some one-hundred amino acids in between the G1 and G2 regions. The G2 region of the 410 amino acid protein Ypt11p lacks a phenylalanine which is conserved throughout the rest of the protein family, in this position it contains an isoleucine instead. The Ypt11 protein was shown to bind GTP. Its intrinsic GTPase activity has not been determined precisely but it was not significantly different from other Ypt proteins when assayed by thin-layer chromatography of ^{32}P -labeled nucleoside phosphates (Lazar *et al.*, 1997; R. Peng, S. Albert and D. Gallwitz, unpublished). Perhaps the additional amino acids change the protein's structure in such a way that the formation of the binding cleft and the contacts holding the nucleoside phosphates in place is disturbed or made impossible. If this be the case, it would follow that Ypt11p is no transport GTPase at all and might have adopted a new and deviant role. Deletion or disruption of the *YPT11* gene does not kill the cell, and under all conditions tested the gene is dispensable. By radioactive labelling of the cell's proteins, the fate of the vacuolar hydrolytic enzymes CPY and alkaline phosphatase (ALP) was followed, and no defects in their processing could be detected. Ypt11p can therefore not claim a place among the Vps group of Ypt proteins (R. Peng and D. Gallwitz, unpublished). The recurring theme of redundancy might offer a hypothesis for the origin of this deviant Ypt protein: True redundancy would render supernumerable genes prone to accumulating mutations that may eventually cause a loss or alteration of function. Evolutionary drift could cause it to assume a different role. Perhaps Ypt11p is a victim of this fate.

7. HOW FEW Ypt GTPASES ARE ENOUGH?

7.1. Essential and Nonessential Ypt GTPases

As discussed above, some Ypt GTPases are obviously more important than others. Under natural growth conditions, the yeast cell will face all kinds of adverse environmental conditions like grossly varying temperatures, changing osmotic conditions or shortages in certain nutrients. With a generation time of—under optimal conditions—about ninety minutes, the fast growing baker's yeast employs the evolutionary strategy of all single-celled microorganisms: maximizing the procreational capacity. Yeast cells are leading an existence under enormous evolutionary pressure. We can thus assume that its genetic contents have been streamlined to a large degree. While the loss of either Ypt1p, Sec4p or Ypt31p/Ypt32p results in lethality, loss of one or even more of the remaining Ypt proteins is tolerable or even without noticeable consequence under laboratory conditions. Why should the forward transport secretory route have a higher priority than any other route? A yeast cell, like any other cell and even viruses, maintains itself and grows to produce more of its kind. When circumstances are favourable and the yeast cell sets out to create a new generation of yeast cells it starts by selecting a bud site, a process which is involving another class of GTP-binding proteins, the Rho/Cdc42-family members (Hall, 1997). Once this decision has been made the cell sets out to deliver vesicles to this site of growth. These vesicles for instance contain polysaccharides to be included in the cell wall of the forming daughter cell or enzymes which break down the mother's cell wall. As was made clear in this review, trafficking of these vesicles depends on Ypt proteins. This reminded, all things fall into place: If Ypt1p is not there, vesicles which have budded off the ER accumulate in the inter-organelle space and enzymes of the extracellular space will not get across the very first threshold. If Ypt31p and Ypt32p fail to fulfill their duty, vesicles will not be set on their tracks to the plasma membrane. If Sec4p happens to be out of order, transport vesicles are no longer able to fuse with the plasma membrane. Thus, disabling forward transport results in a failure to perform cell division and ultimately in a lack of offspring.

It is much more of a challenge to explain why there was no counterselection for the other Ypt proteins. While there seems to be a certain degree of redundancy in the system of vesicular transport, a minimalist microbe should maintain only the genes it really needs to stay alive and multiply successfully. This view is supported by the observation that disruption of many of the non-essential Ypt genes involved in vacuolar transport results in growth defects at elevated temperatures or under osmotic stress. The

seemingly redundant functions will probably turn out to have a well-founded justification under wild-life conditions and will confer upon its possessor a greater evolutionary fitness. In fact, a general contribution of nonessential genes to fitness has recently been experimentally verified in yeast (Thatcher *et al.*, 1998).

7.2. Redundancy among Ypt GTPases

Ypt31p and Ypt32p are phenotypically redundant GTPases. The *YPT32* gene resides on the short arm of chromosome VII while its brother gene *YPT31* acts from its position on the long arm of chromosome V. While it is impossible to tell which might be the more ancient of the two there is no doubt that they have diverged after the chromosomal region into which the ancestral one was embedded got duplicated and translocated to the other chromosome. For the extremely high degree of similarity of the *YPT31/YPT32* gene couple two explanatory hypotheses can be offered: either there is a high evolutionary pressure to retain both variants in the cell or the duplication/translocation event has taken place relatively recently in evolutionary terms. The combined indispensability of the gene pair is difficult to explain: The possibility that they serve a vital role in forward transport of material to be secreted is not a sufficient explanation, since it would require spare copies for instance of the *YPT1* gene as well, and in fact of all other essential genes.

Apart from the *YPT31/YPT32* gene pair, there is a second example of *YPT* genes being subject to genome duplication: The strikingly similar *YPT51* and *YPT53* genes also seem to have diverged from a common ancestor gene (as may have the entire superfamily of *RAS*-like genes from a common primeval proto-*RAS* gene): The *YPT53* gene is found on the long arm of chromosome XIV, in close proximity to the *RAS2* gene with only two other genes between them. On the long arm of chromosome XV we find the *YPT51* gene and—moving three genes further up— the *RAS1* locus. This example gives an idea of how evolution works by shuffling around duplicated parts of chromosomes which then undergo divergent evolution by accumulating mutations. Many more relationships like these will without doubt be found upon closer inspection of chromosomes and whole genomes of different species (see, for example Field and Field, 1997, for a discussion of *Trypanosoma brucei* Ypt/Rab-GTPases and genome duplication). Interestingly, the evolutionarily distant fission yeast contains only one copy of *YPT3*, an essential gene homologous to *YPT31/32*, and one non-essential homologue of *YPT51/52/53* that is involved in endocytic trafficking (Armstrong *et al.*, 1994; Armstrong *et al.*, 1993; Miyake and Yamamoto, 1990). In addition, fission yeast contains a homologue to the animal Rab4 protein,

Ypt4p, for which there is no counterpart in baker's yeast (Bone *et al.*, 1998). How this diversification of the Ypt GTPase family among fungal species leading essentially identical life styles relates to the subtleties of their specializations after a long period of independent evolution is at this moment completely in the dark.

7.3. Ypt GTPases and Vesicular Trafficking Routes

It has been pointed out above that yeast can be seen as a minimalist organism capable of biosynthetic trafficking and able to secrete and endocytose. Ypt/Rab proteins have been identified in mammals, higher plants, unicellular algae, fungi, and protozoan species (Novick and Zerial, 1997; Ueda *et al.*, 1996; Kim *et al.*, 1996; Fabry *et al.*, 1995; Fraga and Hinrichsen, 1994; Bush *et al.*, 1993; Fabry *et al.*, 1993). What makes *Saccharomyces cerevisiae* a suitable model organism to assess the minimal complement of Ypt GTPases a eukaryotic cell needs? Clearly, there are functions like transcytosis or regulated secretion a unicellular organism does not feature. However, these are specialisations of higher eukaryotic cells that give testimony to a different survival strategy. The ability of mammalian genes to complement yeast mutations and the strikingly similar functions of many mammalian homologues of yeast proteins involved in vesicular trafficking demonstrate that comparisons between yeast and higher organisms are actually valid. The complete sequencing of the yeast genome has revealed a similar number of (partially redundant) Ypt GTPases (eleven) and syntaxin-like t-SNAREs (eight) (Götte and Fischer von Mollard, 1998; Holthuis *et al.*, 1998a). Based on the idea that Ypt proteins are key regulators of transport vesicle docking, we will try to determine the minimal amount of vesicular trafficking steps a eukaryotic cell needs (Figure 3): Ypt1p plays a role in docking of ER-derived vesicles with the *cis*-Golgi compartment and might also be involved in transport between early Golgi-compartments. The t-SNARE Sed5p mediates the fusion event at these transport step, whereas the t-SNARE Ufe1p has been described as the syntaxin mediating retrograde transport from the Golgi-apparatus to the ER (Lewis *et al.*, 1997). It is not known if a Ypt GTPase functions in retrograde trafficking between the Golgi and the ER or between Golgi compartments. In the latter case, defects in anterograde or retrograde transport are likely to result in similar phenotypes. Therefore, the role of the GTPases Ypt31p and Ypt32p can be ascribed to intra-Golgi-transport, but a directionality cannot be given. Docking of vesicles to the plasma membrane is mediated by the Sec4 protein, while the subsequent fusion event depends on the Sso1p and Sso2p syntaxins. The recycling of proteins from endosomes to the TGN appears to be dependent on Ypt6p. A similar role has been

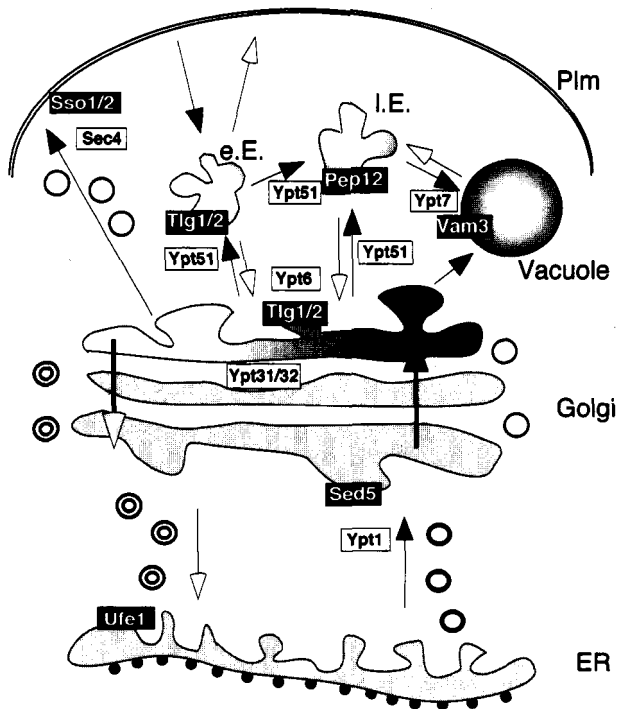


FIGURE 3. Vesicular protein transport pathways and known sites of action of Ypt GTPases (white symbols) and yeast syntaxins (black symbols). Filled arrows indicate forward transport while open arrows indicate retrograde trafficking. Ypt GTPases and syntaxins involved in secretion are essential proteins, whereas loss of the endocytic Ypt proteins or all syntaxins of the endocytic/VPS pathway (Holthuis *et al.*, 1998b) does not result in lethality. e.E. = early endosome, I.E. = late endosome, Plm = plasma membrane.

ascribed to the t-SNARE Tlg2p (Holthuis *et al.*, 1998a), however, this protein might also be involved in endosome biogenesis (Seron *et al.*, 1998). In both cases, interpretation of the data is complicated by the rather subtle phenotype of the *tlg2* gene disruption. Docking to the prevacuolar compartment is regulated by Ypt51p and might in addition be influenced by the other members of the Ypt5p sub-family. The syntaxin-like Pep12-protein has been shown to act at the same transport step (Becherer *et al.*, 1996). Vesicle docking and fusion at the vacuole are mediated by Ypt7p and Vam3p, respectively (Nichols *et al.*, 1997). The observation that, upon high expression, Pep12p and Vam3p can functionally replace each other is most likely due to a saturation of the sorting machinery under these

experimental conditions (Darsow *et al.*, 1998; Darsow *et al.*, 1997; Götte and Gallwitz, 1997).

Having followed the pathways from the ER via the Golgi apparatus to the plasma membrane and to the vacuole, respectively, one fundamental transport route is missing in our picture of intracellular vesicle transport: The docking and fusion of endocytic vesicles with an early endosomal compartment. It has been speculated that the Tlg1p syntaxin is involved in this transport event (Holthuis *et al.*, 1998a), yet no GTPase has been assigned to this pathway. The Ypt10p/Npt11p or one of the Ypt5 proteins are candidates for this transport step, or for an alternative endocytic route that involves the Tlg1p and Tlg2p t-SNAREs (Holthuis *et al.*, 1998b). Given that both a syntaxin-related t-SNARE and a Ypt-GTPase mediate every transport event along the fundamental vesicular transport routes, we have thus succeeded in a rough mapping of all members of these protein families in baker's yeast. While the participation of some Ypt proteins and syntaxins in specific transport steps is well-established, the contribution of others is speculative. Refined methods of analysis, in particular concerning retrograde trafficking and endosomal transport events, will help to obtain a more clear picture of vesicular protein transport in a prototypic eukaryote in the near future.

In summary, three Ypt GTPases suffice in regulating trafficking from the ER to the plasma membrane (Ypt1p, Ypt3p, Sec4p), while three proteins mediate vesicular transport on the endocytic pathway (Ypt51p, Ypt6p, Ypt7p, see also Figure 3). Three Ypt proteins could be redundant (one of Ypt31p/Ypt32p, Ypt52p and Ypt53p), with the restrictions in mind which were made concerning the term redundancy (see section 7.2). For the most recently described GTPases Ypt10p and Ypt11p no function at a defined vesicular transport step has been demonstrated, yet. Over 40 Rab proteins have been identified in mammalian cells (Novick and Zerial, 1997). A comparison of the roles of Ypt and Rab-proteins shows that Ypt1p/Rab1p, and Ypt51p/Rab5p are functionally identical (see Lazar *et al.*, 1997 for discussion). Sec4p/Rab8p and Ypt7p/Rab7p act at the same transport steps and are likely to be functional homologues, too (Feng *et al.*, 1995; Huber *et al.*, 1993). Slightly deviant roles, however are seen in the case of the structural homologues YptGp/Rab6p and Ypt31/32p vs. Rab11a/b (Chen *et al.*, 1998; Mayer *et al.*, 1996; Ullrich *et al.*, 1996; Martinez *et al.*, 1994). These differences might be a sign of functional diversification in the cells of higher eukaryotes: The increase in the number of Ypt/Rab proteins seen between yeast and mammals allowed some of these proteins to adopt functions unique to cells in multicellular environments (e.g. trafficking to select membrane domains in polarized cells), to establish tissue-specific isoforms, and in general a more refined regulation of vesicular transport.

ACKNOWLEDGEMENTS. Work done in the author's laboratory was supported by the Max-Planck-Society, the Deutsche Forschungsgemeinschaft, and the Fonds der Chemischen Industrie. We thank members of the Gallwitz laboratory for discussions and for communicating unpublished results.

Abbreviations Used in This Manuscript

aka	also known as
ALP	alkaline phosphatase
cAMP	cyclic adenosine monophosphate
COPI/II	coatamer protein complexes I/II
ER	endoplasmic reticulum
GAP	GTPase activating protein
GDI	guanine-nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GGT II	geranylgeranyl transferase II
GTP	guanosine triphosphate
NSF	N-ethyl-maleimide sensitive fusion protein
PrA	proteinase A
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
TGN	<i>trans</i> -Golgi network
VAM	vacuolar aberrant morphology mutant
VPS	vacuolar protein sorting mutant
vs.	versus

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Chapter 5

Possible Roles of Long-chain Fatty Acyl-CoA Esters in the Fusion of Biomembranes

Nils Joakim Faxgeman*, Tina Ballegaard,
Jens Knudsen, Paul N. Black,
and Concetta DiRusso

1. INTRODUCTION

Regulation and coordination of membrane trafficking is a central requirement for secretion, endocytosis, cell division and transport between intracellular compartments. The molecular mechanism by which transport vesicles bud from a donor compartment, are transported, recognized and fused to the target membrane has until recently been enigmatic. Ever since Palade (1975) formulated a general outline of the secretory pathway in pancreatic exocrine cells, scientists have been attracted to the study of trafficking and sorting of proteins through the central organellar systems. Shared among all eukaryotic cells, this pathway defines the route of

NILS JOAKIM FÆRGEMAN, PAUL N. BLACK, and CONCETTA DIRUSSO Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, New York 12208, USA. **TINA BALLEGAARD and JENS KNUDSEN** Institute of Biochemistry, Odense University, 5230 Odense M, Denmark. *Corresponding authors.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

transport by which secretory proteins are delivered from the endoplasmic reticulum (ER), through the Golgi apparatus, and to the plasma membrane. The series of fission, transport and fusion events are initiated by the targeting of proteins to the ER accomplished by specific signal sequences (Blobel and Dobberstein, 1975). Export from the ER that involves formation of transport vesicles are programmed to fuse with the next compartment along the pathway. These will eventually fuse with the plasma membrane and release their stored cargo. The cargo can include a diverse array of mediators such as hormones, enzymes and neurotransmitters. The entire process takes about 5 minutes in most cells and is highly regulated. Perturbations in the regulation of this process can result in protein mislocalization and in some cases detrimental diseases. Therefore, understanding the molecular mechanism of these transport events will help in elucidating the causes and treatment of these malfunctions.

The establishment of cell-free systems to study intraorganellar membrane trafficking has led to the identification of a number of proteins and cofactors involved in the general processes of vesicle budding and fusion (Fries and Rothman, 1980; Balch *et al.*, 1984a), and to elucidation of their specific role(s) in specific transport steps. While coat proteins, such as coatamer and the ADP ribosylation factor, ARF, are involved in sculpturing and budding the vesicle from the donor membrane, vesicle- and target-specific receptors (v-SNAREs and t-SNAREs) are involved in docking the vesicle to the target membrane. NSF and SNAP proteins help in fusing the docked vesicle (see below).

Recently, it was found that long-chain (C_{14} – C_{18}) fatty acyl-CoA esters (LCACoA) are essential cofactors in the budding as well as in the fusion of transport vesicles. Beside serving as an important intermediate in lipid biosynthesis and in fatty acid degradation, long-chain fatty acyl-CoA esters have been found to exert a plethora of effects on cellular processes in both inhibitory and stimulatory ways. More than three decades ago acyl-CoA esters were suggested to act as key regulators of fatty acid synthesis (Lynen, 1963) and since then LCACoA esters have been found to affect ion channels, ion pumps, translocators, enzymes, and transcriptional regulation (Table 1). Serving as a substrate in acylation of proteins, LCACoA play a vital role in several signal transduction pathways. In the present article we highlight the numerous effects of long-chain fatty acyl-CoA esters have, with special focus on the putative regulatory effect of long-chain fatty acyl-CoA esters on the fission and fusion of biomembranes. Furthermore, the significance of these effects are discussed in relation to the physiological concentration of long-chain fatty acyl-CoA esters and their binding proteins.

Table 1
Proposed effects of long-chain acyl-CoA esters in cellular regulation and signal transduction. Modified from Færgeman and Knudsen (1997)

Acyl-CoA regulation of	Effect	References
Lipid metabolism		
Fatty acid synthesis:		
Acetyl-coA carboxylase (ACC)	Inhibitory, $K_i = 5.5$ nM	Ogiwara <i>et al.</i> , 1978
AMP-activated kinase-kinase	Stimulatory, 50–200 nM range	Carling <i>et al.</i> , 1987
Acyl-CoA synthetase (ACS)	Inhibitory, $K_i = 4$ μ M	Pande, 1973
Citrate transporter	Inhibitory, $IC_{50} = 32$ μ M	Halperin <i>et al.</i> , 1972
TG/PL/CE synthesis:		
HMG-CoA reductase	Inhibitory, $IC_{50} = 0.16$ – 1.9 μ M	Lehrer <i>et al.</i> , 1981
β-oxidation:		
Carnitine palmitoyltransferase-I (CPT-I)	Inhibitory, $IC_{50} \approx 20$ μ M	Murthy and Pande, 1987a
Medium-chain Acyl-CoA dehydrogenase	Inhibitory	Powell <i>et al.</i> , 1987
CE/TG hydrolysis:		
Hormone sensitive lipase (HSL)	Inhibitory, $IC_{50} = 0.1$ μ M	Jepson and Yeaman, 1992
Energy metabolism		
Adenine nucleotide translocase (ANT)	Inhibitory, $K_i = 1$ μ M	Woldegiorgis <i>et al.</i> , 1981
Glucokinase	Inhibitory, $K_i = 1.8$ μ M	Tippett and Neet, 1982a,b
Glucose-6-phosphatase	Inhibitory, $IC_{50} = 50$ μ M	Fulceri <i>et al.</i> , 1995
Pyruvate dehydrogenase	Inhibitory, $IC_{50} = 30$ μ M	Moore <i>et al.</i> , 1992
Signal transduction		
Ion channels/pumps:		
Ca^{2+} -release from sarcoplasmic reticulum	Stimulatory, $EC_{50} = 6$ μ M	Fulceri <i>et al.</i> , 1994
Ca^{2+} -release from sea urchin eggs	Stimulatory	Chini and Dousa, 1996
GTP-dependent Ca^{2+} -release from ER	Inhibitory, $IC_{50} = 0.5$ μ M	Comerford and Dawson, 1993; Rys-Sikora <i>et al.</i> , 1994
Ca^{2+} -release from ER/ IP_3 -insensitive pool	Stimulatory, $EC_{50} = 50$ μ M	Fulceri <i>et al.</i> , 1993
Ca^{2+} -uptake by Ca^{2+} -ATPases (pumps)	Stimulatory, $EC_{50} = 0.5$ μ M	Deeney <i>et al.</i> , 1992
ΔX and Mg^{2+} content of mitochondria	Inhibitory	Siliprandi <i>et al.</i> , 1992
Plasma membrane Na^+/K^+ -ATPase	Stimulatory, $EC_{50} = 3$ μ M	Kakar <i>et al.</i> , 1987
pH-dependent anion-conducting channel	Inhibitory, $IC_{50} = 2.4$ μ M	Halle-Smith <i>et al.</i> , 1988
ATP-sensitive K^+ -channel	Stimulatory	Larsson <i>et al.</i> , 1996
Protein kinase C (PKC)-subtypes:		
Ca^{2+} /PL/DAG-dependent PKC	Stimulatory, $EC_{50} \approx 15$ μ M	Bronfman <i>et al.</i> , 1988
PKC α (neutrophils)	Inhibitory, $IC_{50} < 10$ μ M	Majumdar <i>et al.</i> , 1991
Gene expression:		
Nuclear thyroid hormone receptor	Inhibitory, $K_i = 0.45$ μ M	Li <i>et al.</i> , 1990; 1993
HNF4 α transcription factor	Stimulatory and inhibitory	Hertz <i>et al.</i> , 1998
FadR, <i>E. coli</i> transcription factor	Inhibitory, $K_m = 12.1$ nM	Raman and DiRusso, 1995
<i>OLE1</i> gene expression (yeast)	Repression (Δ -9 unsaturated)	Choi <i>et al.</i> , 1996
<i>ACC</i> gene expression (yeast)	Repression	Kamiryo <i>et al.</i> , 1976
Protein sorting:		
Vesicular transport in Golgi, budding	Stimulatory, low μ M-range	Ostermann <i>et al.</i> , 1993; Pfanner <i>et al.</i> , 1989
Vesicular transport in Golgi, fusion	Stimulatory, low μ M-range	Pfanner <i>et al.</i> , 1990
Homotypic vacuole fusion (yeast)	Stimulatory, 0.1–1 μ M	Haas and Wickner, 1996
GTP-dependent vesicle fusion in ER	Inhibitory, $IC_{50} = 15$ – 18 μ M	Rich <i>et al.</i> , 1995; Comerford and Dawson, 1993
Proteolysis:		
Proline endopeptidase	Inhibitory, $K_i \approx 9$ μ M	Yamakawa <i>et al.</i> , 1990

2. BIOPHYSICAL PROPERTIES OF LONG-CHAIN FATTY ACYL-COA ESTERS AND THEIR INTERACTION WITH BIOMEMBRANES

LCACoA esters are capable of interacting with biological membranes due to the amphipathic nature of this molecule. Like other detergents, long-chain fatty acyl-CoA esters form molecular solutions at low concentrations, but as the concentration increases the critical micelle concentration is reached and micelle formation is favored. Above the critical micelle concentration, the concentration of free molecules in equilibrium with micelles remains essentially the same and independent of the total concentration of acyl-CoA (Constantinides and Stein, 1985). Employing a number of biophysical techniques, the critical micelle concentration of palmitoyl-CoA has been estimated to be in the range of 30–80 μM (Constantinides and Stein, 1985; Powell *et al.*, 1981). Despite the micellar properties of long-chain fatty acyl-CoA esters, regulation of cellular metabolism and signal transduction by these compounds seems to be specific and direct. Not only are many metabolic pathways inhibited when concentrations are well below the critical micelle concentration, but the existence of intracellular micelles is unlikely due to the high partitioning coefficient favoring long-chain fatty acyl-CoA ester interaction with various membranes within the cell. Long-chain fatty acyl-CoA esters readily partition into biomembranes by insertion of their hydrophobic acyl-chain into the phospholipid bilayer (Requero *et al.*, 1995a,b; Peitzsch and McLaughlin, 1993; Boylan and Hamilton, 1992). The association constant of palmitoyl-CoA for the interaction with phospholipid membranes has been determined to be approximately $1.5\text{--}5 \times 10^5 \text{M}^{-1}$ (Requero *et al.*, 1995a; Peitzsch and McLaughlin, 1993). The partition constant strongly depends on the acyl-chain length (Requero *et al.*, 1995a; Peitzsch and McLaughlin, 1993; Boylan and Hamilton, 1992) but is nearly independent of the type of polar-head group, indicating that the acyl-group partitions into the membrane while the polar head group is located on the membrane surface in the aqueous environment (Peitzsch and McLaughlin, 1993; Boylan and Hamilton, 1992). However, using infrared spectroscopy it has recently been shown that part of the CoA moiety may participate in forming polar interactions with the phospholipid carbonyl groups at the membrane surface (Echabe *et al.*, 1995). No transbilayer movement of oleoyl-CoA is observed, even when oleoyl-CoA was pre-incubated with phosphatidylcholine vesicles for up to 24h (Boylan and Hamilton, 1992).

Partitioning of long-chain fatty acyl-CoA esters into membranes markedly perturbs the integrity of phospholipid bilayers (Boylan and Hamilton, 1992; Banhegyi *et al.*, 1996). Palmitoyl-CoA was found to

decrease the latency of β -glucuronosyltransferase in rat liver microsomes and increased the permeability of microsomal vesicles to sucrose and citrate (Banhegyi *et al.*, 1996). Boylan and Hamilton (1992) have shown that >15 mol % oleoyl-CoA disrupts the bilayer structure of phosphatidylcholine vesicles and causes production of mixed micelles of phospholipids and long-chain fatty acyl-CoA esters. Compared to fatty acids and lysophospholipids, long-chain fatty acyl-CoA esters were found to be the most potent in disruption of membrane bilayers (Boylan and Hamilton, 1992). However, only acylcarnitines, and not long-chain fatty acyl-CoA esters, are able to impair the membrane permeability barrier and produce complete membrane solubilization (Requero *et al.*, 1995a,b; Echabe *et al.*, 1995; Goñi *et al.*, 1996).

Long-chain fatty acyl-CoA esters partition into microsomal membranes *in vitro* (Juguelin *et al.*, 1991; Rasmussen *et al.*, 1990; Polokoff and Bell, 1978). However, the majority of the bound acyl-CoA esters were hydrolyzed within the first hour of incubation (Juguelin *et al.*, 1991) indicating that long-chain fatty acyl-CoA esters are unlikely to accumulate in biological membranes under *in vivo* conditions.

3. VESICLE TRAFFICKING

3.1. Assembly of Transport Vesicles

Insights into the molecular mechanism of the assembly and disassembly of transport vesicles within the Golgi apparatus were initially obtained by measuring transport of vesicular stomatitis virus (VSV)-encoded G-protein from a donor to an acceptor Golgi membrane population (Balch and Rothman, 1985; Balch *et al.*, 1984a). In these experiments, the donor population of Golgi harboring the VSV-encoded G-protein and an acceptor population of Golgi containing a glycosylating enzyme are incubated together with a $100,000 \times$ G cytosolic fraction, an energy source and radiolabeled N-acetyl glucosamine. Transfer and glycosylation of the VSV-G protein from the donor to the acceptor membrane is then measured by immunoprecipitation. The results of these experiments show that the transport process is a vectorial process: VSV G-protein moves unidirectionally across the Golgi compartments (Braell *et al.*, 1984; Rothman *et al.*, 1984a,b; Dunphy *et al.*, 1981). Glycosylated G-protein only resides in the acceptor Golgi population, indicating that the transport process involves a diffusible transport intermediate (Braell *et al.*, 1984). Reconstitution of intra-Golgi transport revealed that 70 nm diameter vesicles are formed, which contain the VSV G-protein. Formation of the vesicle occurs in an ATP- and cytosol-dependent manner (Balch *et al.*, 1984b). The majority of the vesicles formed

and the entire population of newly budded vesicles carries an 18nm coat on the surface which is distinct from clathrin coated endocytic vesicles (Goldstein *et al.*, 1979) since they lack the well-defined geometry of the clathrin coat. These are referred to as a non-clathrin coated vesicle. Using inhibitors of transport, two distinct forms of transport vesicles can be seen by electron microscopy. Treatment of a nonhydrolyzable analogue of GTP, such as GTP- γ -S, results in accumulation of vesicles enshrouded in a dense protein coat (Melancon *et al.*, 1987). Transport is also blocked by the cysteine-alkylating reagent N-ethylmaleimide, NEM, (Glick and Rothman, 1987), but results in accumulation of uncoated vesicles (Malhotra *et al.*, 1988). The fact that GTP- γ -S and NEM give rise to accumulation of coated vesicles, demonstrates that coated vesicles precedes uncoating of the transport vesicles (Orci *et al.*, 1989). The nature of the coat was revealed by purification of coat proteins from vesicles formed from Golgi fractions incubated with GTP- γ -S (Malhotra *et al.*, 1989; Serafini *et al.*, 1991a). The coat proteins, COPs, consist of a number of major components (also called coatomer) which are externally disposed. These include α -COP, β -COP, β' -COP, γ -COP, δ -COP, ϵ -COP, ζ -COP, and a small GTP-binding ras-like protein called ARF, ADP-ribosylation factor. These proteins have molecular masses very similar to the proteins composing the clathrin coat. Sequence analysis showed that β -COP is weakly homologous to the clathrin coat protein, β -clathrin (Duden *et al.*, 1991; Serafini *et al.*, 1991b). ARF was initially described as a cofactor required for ADP-ribosylation of a trimeric G-protein by cholera toxin (Kahn and Gilman, 1986). Since the coat is virtually absent from Golgi membranes prior to transport and because coatomers readily can be purified from cytosol, it is suggested that coated vesicles are assembled from a dispersed pool of cytosolic subunits at the Golgi membrane surface (Balch *et al.*, 1992; Orci *et al.*, 1991; Serafini *et al.*, 1991a). Further support for the role of coatomers in vesicle formation come from studies using the natural fungal metabolite brefeldin A that causes secretion blockade and redistribution of Golgi into the ER (Klausner *et al.*, 1992). Brefeldin A was found to prevent association of β -COP and ARF with Golgi membranes due to the inhibition of the exchange of ARF bound GDP with GTP (Donaldson *et al.*, 1992a; Helms and Rothman, 1992). Thus, by preventing ARF binding to Golgi membranes, the association of coatomers is restrained from binding to membranes and hence vesicle formation is hampered. Moreover, coatomer depleted cytosol does not stimulate the formation of coated vesicles from Golgi membranes *in vitro*. However, upon addition of purified coatomer coated vesicles were formed substantiating the role of coatomer in the budding process (Orci *et al.*, 1993a,b).

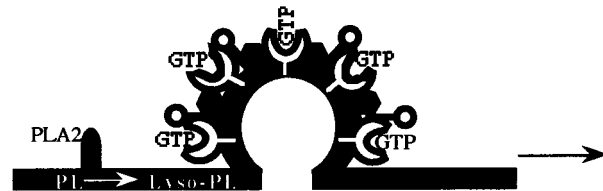
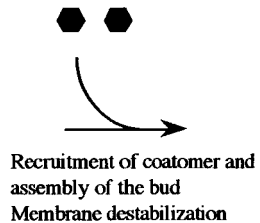
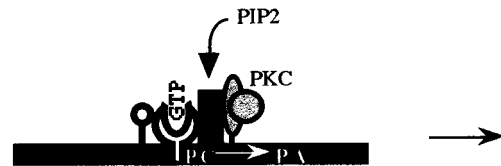
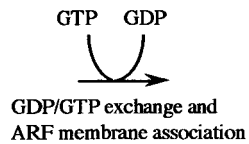
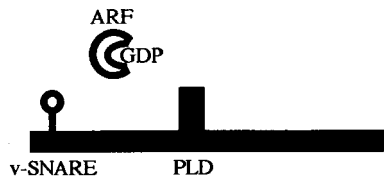
The identification of ARF as a coat protein led to development of a

model that describes how budding and uncoating is triggered. The model predicts that ARF, which is N-myristylated (Kahn *et al.*, 1991), is water-soluble when GDP is bound. Upon GDP/GTP exchange a conformational switch exposes the myristoyl-group and ARF is inserted into the lipid bilayer. Upon association with the Golgi membrane, ARF recruits coatamer from the cytosol and the bud assembles (Palmer *et al.*, 1993; Donaldson *et al.*, 1992b). Non-myristylated ARF can undergo nucleotide exchange but is not membrane associated. Apparently, membrane budding is driven by coatamer binding and assembly; the role of ARF is to initiate budding by serving as platform for coatamers.

3.2. Fusion of Transport Vesicles

The newly formed vesicle must be able to recognize and fuse with its target membrane once the vesicle is pinched off. Uncoating is dependent on GTP hydrolysis since a mutated version of ARF that is impaired in GTP-hydrolysis but not binding cannot trigger uncoating. This inhibits transport and accumulation of coated vesicles can be observed (Tanigawa *et al.*, 1993), which are incapable of fusion with its target Golgi membrane (Ostermann *et al.*, 1993). This confirms that uncoating of the vesicle is a prerequisite for subsequent fusion.

Reconstitution of vesicle trafficking in the presence of NEM was observed to result in accumulation of uncoated vesicles. This implies that an NEM-sensitive fusion protein (NSF) participates in the docking and fusion between the vesicle and target membrane. Subsequently, NSF was isolated and found to be an ATPase required for vesicle and membrane fusion (Lenzen *et al.*, 1998; Matveeva and Whiteheart, 1998; Whiteheart *et al.*, 1994; Block *et al.*, 1988; Malhotra *et al.*, 1988). NSF participates in membrane fusion in many steps along the pathway from ER to the plasma membrane and for endocytosis in both animals and yeast (Hay and Scheller, 1997; Beckers *et al.*, 1989; Diaz *et al.*, 1989). NSF is a homooligomeric protein consisting of three 76kDa subunits. The protein binds to Golgi membranes in an cytosol-dependent manner (Weidman *et al.*, 1989). Further investigation led to the identification of Soluble NSF attachment proteins, named SNAPs (Clary and Rothman, 1990), of which α -, β -, and γ -SNAP have been purified from the cytosol (Whiteheart *et al.*, 1993; Clary *et al.*, 1990). While α -, and γ -SNAP are ubiquitously expressed, β -SNAP seems to perform a brain-specific function (Whiteheart *et al.*, 1993; Schiavo *et al.*, 1995). α - and β -SNAP share the highest degree of homology (83%), while γ -SNAP is more distantly related (Whiteheart *et al.*, 1993). The SNAP proteins bind to Golgi membranes in the absence of NSF, arguing that membrane association of SNAPs precedes NSF membrane binding (Whiteheart



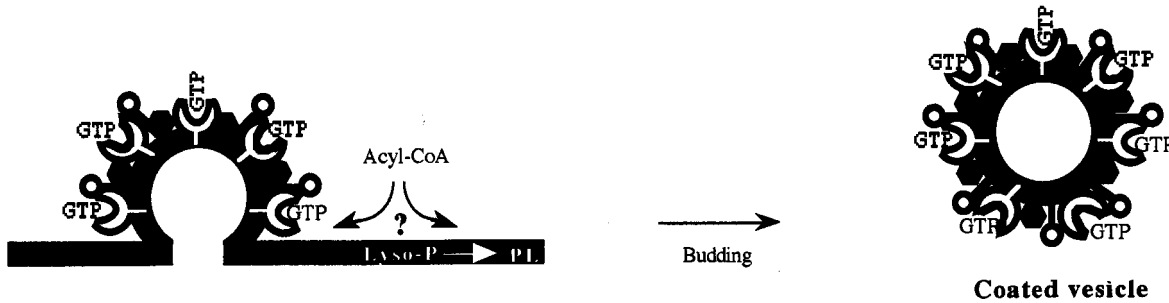


FIGURE 1. Assembly and budding of transport vesicles. Upon GDP/GTP exchange ARF binds to membranes via its N-myristoyl group and recruits coatamer and the bud assembles. G-proteins and remodeling of phospholipids may play important roles in this process. Acyl-CoA esters are proposed to be involved in pinching off the bud.

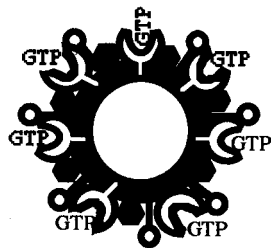
et al., 1992). Using a NSF-SNAP affinity column, the first three SNAP receptors (SNAREs) were purified from detergent-solubilized brain extracts. These are Synaptobrevin/VAMP, syntaxin, and Synaptosome-Associated Protein-25 kDa (SNAP-25), respectively (Söller *et al.*, 1993b). NSF, SNAPs and SNAREs form a 20s fusion particle which can be isolated from intact Golgi. The 20s fusion particle has been suggested to form a general fusion machine only stable in the absence of hydrolyzable ATP (Wilson *et al.*, 1992). Due to its localization in the synaptic vesicle (Baumert *et al.*, 1989; Trimble *et al.*, 1988), VAMP is called a v-SNARE, while syntaxin and SNAP-25 found in the pre-synaptic membrane (Bennett, 1995; Oyler *et al.*, 1989) are called target SNAREs, or t-SNAREs. Therefore, VAMP, syntaxin, and SNAP-25 are the SNAREs of the nerve terminal. In contrast to VAMP and syntaxin, SNAP-25 does not span the membrane but anchored to the presynaptic plasma membrane through a covalently bound palmitoyl group in the C-terminus (Gonzalo *et al.*, 1998; Veit *et al.*, 1996; Hess *et al.*, 1992).

Since SNAPs and NSF can not provide the specificity observed in membrane docking and fusion, Söller *et al.* (1993a) proposed the SNARE hypothesis which postulates that each membrane involved in NSF dependent membrane fusion contains specific SNAREs, homologues to the synaptic proteins, and that the specificity is provided through pairwise interaction between a v-SNARE, and at least one t-SNAREs. Several v-SNAREs and t-SNAREs have now been identified in yeast, plants, and animals (Linial, 1997; Hey and Scheller, 1997; Bennett, 1995) and are localized selectively to secretory vesicles, ER, Golgi, plasma membranes, endosomes, and nuclear membranes (Bock and Scheller 1997; Lewis *et al.*, 1997; Rothman and Wieland, 1996; Pelham *et al.*, 1995). Docking of vesicles to the acceptor membrane is accounted for by the complementarity between v- and t-SNAREs. Not only are the SNAREs providing the specificity for docking, they also form a platform for the assembly of the general fusion machine, including NSF and SNAPs. The importance of SNAREs in vesicle trafficking is further underlined by the observation that the three synaptic SNAREs are specific targets for the lethal Clostridial toxins (Washbourne *et al.*, 1998; Ahnert-Hilger and Bigalke, 1995; Pellegrini *et al.*, 1995). Tetanus toxin blocks inhibitory neurons in the spinal cord and causes spastic paralysis, while botulinum toxins induce muscle fatigue by inhibiting synaptic transmission at the neuromuscular junction. These toxins function by catalyzing proteolytical cleavage of SNAREs, leading to a block of transmitter release (Goda, 1997; Montecucco *et al.*, 1996).

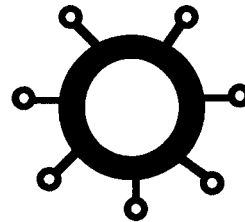
Besides interacting with one another and with the SNAPs, v- and t-SNAREs are also capable of interacting with other important factors involved in membrane fusion. In synaptic vesicle fusion docked vesicles do

not fuse with membranes until triggered by the influx of Ca^{++} . A recently identified v-SNARE, synaptotagmin, is believed to act as a Ca^{++} -responsive gate (Schiavo *et al.*, 1997; Schiavo *et al.*, 1996; Südhof and Rizo, 1996; Schiavo *et al.*, 1995). Synaptotagmin is palmitoylated (Veit *et al.*, 1996) and binds Ca^{++} in a phospholipid dependent manner (Brose *et al.*, 1992). Synaptotagmin interacts with the t-SNARE SNAP-25 and with the brain-specific β -SNAP, thereby recruiting NSF and thus forming a Ca^{++} responsive fusion machinery in close proximity of the target membrane. The membrane fusion event triggered by Ca^{++} may be mediated through specific interactions between v- and t-SNAREs and Ca^{++} -channels. Syntaxin, synaptotagmin, synaptobrevin, and SNAP-25 can together form a complex with N-type Ca^{++} -channels (el Far *et al.*, 1995; Sheng *et al.*, 1994; O'Connor *et al.*, 1993). This is supported by the observations that injection of peptides, which dissociate the Ca^{++} -channel-SNARE complex, into pre-synaptic neurons attenuates synchronous release of transmitter (Mochida *et al.*, 1996) and that α -SNAP is required for Ca^{++} -dependent exocytosis in chromaffin cells (Burgoyne and Morgan, 1998).

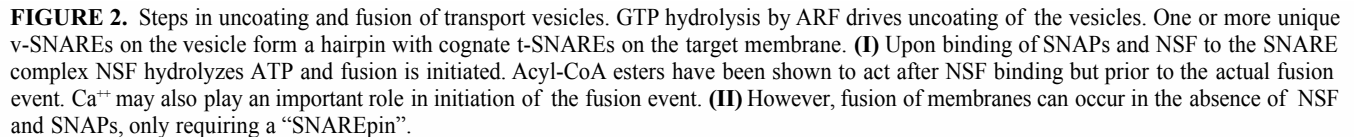
The exact mechanism of how membrane fusion occurs is unknown. Although, SNAPs and NSF are associated with many fusion processes, their precise role in the membrane fusion event is yet to be demonstrated. Several lines of evidence suggest that NSF and SNAPs serve a general function in vesicle trafficking. NSF and SNAPs have been localized to several subcellular compartments by immunocytochemistry (Whiteheart *et al.*, 1993). They are clearly involved as mentioned above in ER to Golgi and intra-Golgi transport; as well as in neurotransmission, transcytosis, homotypic vacuole and endosome-endosome fusion (Apodaca *et al.*, 1996). Upon ATP hydrolysis by NSF the 20S fusion particle dissociates (Hayashi *et al.*, 1995; Söhlner *et al.*, 1993a). This energy-dependent dissociation was proposed to drive fusion of v- and t-SNARE containing membranes. Thus, NSF can be regarded as a chaperone-like protein that provides energy from ATP hydrolysis to reconfigure the structure of the SNARE complex. However, fusion of membranes can occur in the absence of NSF and SNAPs. Fusion in this case, requires only a SNAREpin between v- and t-SNARE. These data suggest that NSF and SNAPs are not involved in the fusion event *per se*, but function to separate the SNARE complex after a round of fusion to allow repeated use of SNAREs in subsequent rounds of fusion (Ungermann *et al.*, 1998; Weber *et al.*, 1998; Nichols *et al.*, 1997; Mayer *et al.*, 1996). It has also been suggested that rearrangement of the SNARE- Ca^{++} -channel complex mediated by NSF-driven ATP hydrolysis makes synaptotagmin more susceptible to Ca^{++} binding, which leads to destabilization of the complex and subsequently membrane fusion (Goda, 1997).



GTP hydrolysis and
uncoating of vesicle



Formation of SNAREpins
between v- and T-SNAREs



4. LONG-CHAIN FATTY ACYL-COA ESTERS AS COFACTORS FOR VESICLE BUDDING AND FUSION

The first evidence of the role for long-chain fatty acyl-CoA esters in vesicular transport was obtained by Glick and Rothman (1987) while searching for sulphhydryl-containing compounds that could serve as controls to show the specific affect of NSF in restoring vesicle transport activity in a cell-free transport assay. Surprisingly, they found that CoASH stimulated the ability of NSF to restore transport activity in NEM-treated membranes. As the major carrier for acyl-groups, the authors suggested that the affect obtained by adding CoASH was due to the formation of acyl-CoA by acyl-CoA synthetases (ACS) present in the Golgi membrane fraction. This proposal was supported by the observation that desulpho CoA is ineffective in replacing CoASH and palmitoyl-CoA stimulates transport activity to a greater extent than CoASH. Each of these results suggest that long-chain fatty acyl-CoA esters are important cofactors in certain steps in vesicular trafficking (Glick and Rothman, 1987). At very low ATP levels where synthesis of long-chain fatty acyl-CoA esters is significantly reduced due to the high K_m of ACS for ATP (2–5 mM), long-chain fatty acyl-CoA esters are able to stimulate transport, further substantiating that long-chain fatty acyl-CoA esters are important cofactors in membrane trafficking. When added to Golgi membranes, the majority of the long-chain fatty acyl-CoA esters are hydrolyzed due to the presence of acyl-CoA hydrolases (Pfanner *et al.*, 1989). Using triacscin C, an inhibitor of acyl-CoA synthetases, it was demonstrated that fatty acids have to be activated to fatty acyl-CoA to stimulate transport activity (Pfanner *et al.*, 1989). Recently, it was demonstrated that acyl-CoA synthetase 1 is associated with GLUT4-containing vesicles (Sleeman *et al.*, 1998). This may provide a mechanism to maintain a high local concentration of LCACoA esters to support vesicle trafficking. While detergents like palmitoylcarnitine, lysolecithin and Triton X-100 inhibit transport (Pfanner *et al.*, 1989; Glick and Rothman, 1987) it could be ruled out that the observed stimulatory effect of palmitoyl-CoA was due to its detergent like properties.

Albeit, these experiments showed for the first time that long-chain fatty acyl-CoA esters are required in the vectorial movement of transport vesicles, it was not until Pfanner *et al.* (1989) used a nonhydrolyzable palmitoyl-CoA analogue in combination with electron microscopy to show that long-chain fatty acyl-CoA esters are required in budding of transport vesicles. As mentioned above, when transport is reconstituted *in vitro* by incubation of Golgi membranes with ATP and cytosol, the number of vesicles and buds increase dramatically as observed by electron microscopy. However, when a nonhydrolyzable palmitoyl-CoA analogue is included, the

formation of coated transport vesicles and buds are greatly reduced (Pfanner *et al.*, 1989). Ethanol has a similar effect, but is reversed by addition of palmitoyl-CoA, suggesting for the first time that budding is acyl-CoA dependent and that an acyl-transfer mechanism is involved in budding of coated vesicles from Golgi cisternae. The *in vitro* transport assay was further refined by removing cytosolic factors from the Golgi membranes by low salt extraction, and it was shown that coated buds can accumulate in a GTP-dependent manner when only purified coatomer and N-myristylated ARF are added (Ostermann *et al.*, 1993). Subsequent addition of palmitoyl-CoA results in pinching off of coated vesicles, revealing that acyl-CoA is sufficient for fusion of the adjoining regions of the lipid bilayer within the coated bud.

Consequently, investigations were conducted to determine whether LCACoA are required in other steps in the intra-Golgi transport process (Pfanner *et al.*, 1990). If acyl-CoA esters only are required in the budding process, then consumption of coated vesicles should be insensitive to addition of the non-hydrolyzable acyl-CoA analogue. A primed donor was formed by preincubating donor membranes in the presence of cytosol, ATP and palmitoyl-CoA. Subsequent addition of acceptor membranes and the non-hydrolyzable acyl-CoA analogue did not lead to fusion between the coated vesicles and the acceptor membrane, whereas preincubation of donor membranes with acceptor membranes completed transport of the coated vesicles. These observations suggested that acyl-CoA esters are required in other steps in the transport pathway, downstream of the budding process (Pfanner *et al.*, 1990). As described above, the role of NSF appears to be associated with the actual fusion event. Using purified NSF and NEM-treated membranes (donor and acceptor) preincubated with cytosol and ATP, it was shown that NSF membrane binding occurs prior to inhibition of fusion by the non-hydrolyzable acyl-CoA analogue, suggesting that acylation is required for maturation of the junction between the transport vesicle and the cisternae. A further detailed kinetic analysis of the acyl-CoA effect identified an NEM-resistant fusion intermediate occurring immediately prior to fusion which is insensitive to the acyl-CoA analogue implying that the step which-ultimately leads to membrane fusion, is independent of acyl-CoA esters (Pfanner *et al.*, 1990).

The final step in vacuole inheritance in *Saccharomyces cerevisiae* is vacuole fusion in daughter cells, allowing yeast to maintain vacuoles as low-copy organelles. As in heterotypic fusion NSF and α -SNAP have also been shown to be required in homotypic vacuole fusion in yeast (Haas and Wickner, 1996). Haas and Wickner (1996) observed that myristoyl-, and palmitoyl-CoA as well as CoASH supported vacuole fusion *in vitro*, while short- and very-long chain acyl-CoA esters did not. Consistent with the

previous observation by Pfanner *et al.* (1990) a desulfo CoA analogue did not stimulate fusion (Haas and Wickner, 1996). The stimulatory effect was dependent on the presence of functional NSF since the effect was completely abolished by preincubation of vacuoles with NSF and yeast α -SNAP antibodies. The maximal stimulatory effect of palmitoyl-CoA was achieved at 1 μ M, similar to the 10 μ M required for optimal stimulation of Golgi transport (Glick and Rothman, 1987), and appeared to be specific since palmitoyl-carnitine was ineffective (Haas and Wickner, 1996). The synergistic effect of LCACoA esters and NSF on vacuole fusion is also consistent with the effect observed in intra-Golgi transport (Glick and Rothman, 1987).

4.1. Palmitoylation of Proteins Involved in Membrane Trafficking

The fact that a non-hydrolyzable palmitoyl-CoA analogue inhibits the process of pinching off the mature transport vesicles led to the obvious suggestion that reversible palmitoylation of at least one component of the maturing transport vesicle is a prerequisite for budding to occur (Ostermann *et al.*, 1993; Pfanner *et al.*, 1989). The pinching off process depends on fusion of adjoining regions of the same lipid bilayer at the base of the bud, initiated from the luminal side of the membrane. Hence, the simplest explanation of the requirement for acyl-CoA esters is that acylation of a protein component facing the luminal side triggers membrane fission as the assembly of the coat drives the luminal surfaces of the lipid bilayer to approach each other. Based on studies of ethanol acyl transferase (Polokoff and Bell, 1978), it was suggested that microsomal membranes can take up LCACoA esters which serve as substrates in an acylation process on the luminal side of the membrane (Ostermann *et al.*, 1993). However, even though LCACoA esters partition into lipid bilayers (see above) this appears to be unlikely since no transbilayer movement of oleoyl-CoA is observed when preincubated with phosphatidylcholine vesicles (Boylan and Hamilton, 1992).

The assembly of the mature transport vesicle requires recruitment of a number of cytosolic proteins to the membrane. Thus, it has also been suggested that membrane targeting and association of such a recruited component could depend on reversible palmitoylation. In mammalian cells a number of cellular processes requiring vesicle formation and membrane fusion including endocytosis, exocytosis, and receptor recycling are inhibited as cells undergo mitosis (Featherstone *et al.*, 1985). Mundy and Warren (1992) identified a 62 kDa Golgi protein (p62) which specifically became palmitoylated when cells entered mitosis from interphase, whereas when cells exited mitosis and entered G1/interphase p62 became deacylated. Brefeldin A, CCCP and monensin each of which inhibit vesicular transport,

primarily at the level of the ER and Golgi stack stimulate palmitoylation of p62 in interphase cells, but have no effect on p62 palmitoylation in mitotic cells (Beckers and Balch, 1989; Kabcenell and Atkinson, 1985; Tartakoff, 1983; Fries and Rothman, 1980). Palmitoylation of p62 occurs in CHO-, normal rat kidney-, A431-, HeLa-, and BHK cells indicating that acylation is important to function (Mundy and Warren, 1992). The authors suggested that p62 represents an acyl-transferase, acting on another factor essential for vesicle maturation. They suggested that the acyl transferase became hyperacylated and trapped in an acylated intermediate when transport was inhibited during mitosis or brefeldin A treatment (Mundy and Warren, 1992). It is noteworthy that a 65 kDa membrane-bound palmitoyl transferase (see below) has been purified and identified in a number of diverse tissues like heart, liver, pancreas, lung, kidney, and salivary glands and shown to reside in the ER and in the Golgi apparatus (Kasinathan *et al.*, 1990).

Acylation of p62 may be required for the binding of coatmer to membranes since p62 is not acylated in PtK1 cells which are resistant to brefeldin A (Mundy, 1995). Aluminum fluoride and mastoparan each activate heterotrimeric G-proteins and each antagonize also the effect of brefeldin A on β -COP binding to Golgi membranes (Ktistakis *et al.*, 1992) and reduce the palmitoylation of p62 (Mundy, 1995). Mastoparan, a peptide that stimulates GTP/GDP exchange in heterotrimeric G-proteins, stimulates binding of β -COP to Golgi membranes but this function is antagonized by pertussis toxin catalyzed ADP-ribosylation of $G\alpha_{31}$ subunits (Ktistakis *et al.*, 1992; Donaldson *et al.*, 1991). p200, a brefeldin A sensitive protein localized on trans-Golgi membranes, accumulates upon treatment with mastoparan, aluminum fluoride or GTP- γ -S (de Almeida *et al.*, 1993). In contrast, Leyte *et al.* (1992) found that mastoparan inhibits vesicle formation in the trans-Golgi network *in vitro*; this effect was abolished by pertussis toxin. It has recently been shown that the α_{31} subunit of heterotrimeric G-proteins is associated with the Golgi complex (Ercolani *et al.*, 1990; Stow *et al.*, 1991). Overexpression or activation of the α_{31} subunit on Golgi membranes inhibits constitutive secretion, whereas uncoupling of the α_{31} subunit by ADP-ribosylation by pertussis toxin stimulates Golgi trafficking (Stow *et al.*, 1991). The $G\alpha_{31}$ subunit may also regulate vesicle transport between the ER and Golgi since a $G\alpha_{31}$ antibody modulates transport between these compartments (Wilson *et al.*, 1993). Besides α_{31} subunits, α_s -subunits have also been localized to the trans-Golgi network (Leyte *et al.*, 1992). Activation of α_s by cholera toxin causes a stimulation of constitutive secretion, but this effect is reversed by activation of α_i with aluminum fluoride or GTP- γ -S (Leyte *et al.*, 1992). These paradoxical findings may suggest that coating of buds in some way is stimulated by activation of $G\alpha_s$ and membrane

fission to produce coated vesicles and this process is suppressed by activation of $G\alpha_{i/o}$.

A number of small ras-related GTP-binding proteins of the rab-family, is also found in association with the vesicle transport machinery and are believed to serve crucial roles in vesicle formation, transport, and fusion with target membranes (Novick and Zerial, 1997; Rothman and Söllner, 1997; Chavrier *et al.*, 1990a,b). In mammalian cells rab1a and rab1b have been shown to be essential in the early steps of vesicle transport. This is in line with the proposed function of the rab1-related yeast ypt1 and sec4 proteins which appear to be important factors in subsequent targeting and fusion of transport vesicles (Segev, 1991; Walworth, 1989; Goud *et al.*, 1988). Rab1a can, when overexpressed, replace Ypt1p function in yeast (Haubruck *et al.*, 1989). Rab1b is located in the ER and in the Golgi apparatus and is required for ER to Golgi transport as well as intra-Golgi transport (Plutner *et al.*, 1991). This function may be due to the association of rab1 to transport vesicles (Schwaninger *et al.*, 1992). Of particular interest is the finding that the ypt1 protein is palmitoylated on at least one of the two cysteines in its C-terminus. The acylation seems to determine the degree of membrane association since the modified protein predominantly resides in the membrane fraction while a ypt1 protein lacking the C-terminal residues is exclusively found in the soluble fraction (Molenaar *et al.*, 1988). Since the C-terminal domain of rab proteins have been suggested to act as targeting signals and is highly conserved (Chavrier *et al.*, 1991a; Molenaar *et al.*, 1988) there is reason to believe that the membrane association and function of rab1, at least in part, also depends on palmitoylation.

It is intriguing that the effects of G-protein modulators parallels the acylation of p62. These observations suggest the regulation of a protein acyl-transferase involved in vesicle assembly and membrane budding by a heterotrimeric G-protein. Dynamic palmitoylation also plays an important role in cycling of heterotrimeric G-proteins between basal and agonist-activated states (reviewed in Mumby, 1997). Palmitoylation of α_s has been shown to increase the affinity for $\beta\gamma$ (Iiri *et al.*, 1996), and when bound to $\beta\gamma$ palmitoylated α_s is more resistant to depalmitoylation by a protein thioesterase (Iiri *et al.*, 1996). Likewise, $\beta\gamma$ stimulates palmitoylation of α_s and α_{11} (Dunphy *et al.*, 1996). The current model predicts that prior to activation $G\alpha$ is palmitoylated, but during activation it becomes deacylated and dissociates from $\beta\gamma$ to modulate the activity of its downstream effectors. GTP-hydrolysis triggers $G\alpha$ to reassociate with $\beta\gamma$, $G\alpha$ is then reacylated and inactivated (Mumby, 1997). Thus, in this regulatory scheme ARF, COPs, heterotrimeric G-proteins and rab proteins operating together with specific palmitoyl transferases may be important factors regulating the process of assembling of and pinching off vesicles.

4.2. The Enzymology of Protein Palmitoylation

Palmitoylation of proteins refers to the posttranslational modification in which palmitic acid is covalently attached to cysteine residues via a thioester bond (Towler *et al.*, 1988; Schultz *et al.*, 1988). Despite the early identification of LCACoA esters as the immediate donor of acyl-groups (Bizzozero and Lees, 1986; Berger and Schmidt, 1984a) and the development of a number of cell-free acylation systems (Gutierrez and Magee, 1991; Mack *et al.*, 1987; Berger and Schmidt, 1984b), there have only been a few protein-acyl transferases described. A number of proteins can be acylated in a cell-free system via a non-enzymatic transacylation reaction between LCACoA esters and thiol-groups on the proteins. These include, for example, rhodopsin (Moench *et al.*, 1994; Oibrien *et al.*, 1987), myelin PO glycoprotein (Bharadwaj and Bizzozero, 1995), myelin proteolipid protein (Ross and Braun, 1988; Bizzozero *et al.*, 1987a), Semliki Forest virus E2 glycoprotein (Berger and Schmidt, 1984b), the α -subunit of certain heterotrimeric G-proteins (Duncan and Gilman, 1996), pulmonary surfactant protein SP-C (Qanbar and Possmayer, 1994) as well as some synthetic cysteine-containing peptides (Bharadwaj and Bizzozero, 1995; Quesnel and Silvius, 1994). The biological significance of autoacylation of proteins is unknown, but it is interesting that the palmitoylated cysteine residues in myelin proteolipid protein modified *in vivo* are also autoacylated *in vitro* and that addition of cell extracts to cell-free autoacylation assays in some cases do not enhance protein acylation (Bizzozero *et al.*, 1987b; Bizzozero and Lees, 1986). However, the observation that the rate of autoacylation *in vitro* is lower than that required in order to sustain the rapid turnover which is observed *in vivo* questions the biological significance of autoacylation (Bizzozero and Good, 1991).

Thioesterification of proteins is a posttranslational event that occurs in the ER, Golgi apparatus or at the plasma membrane. Protein acyl transferase activity has been identified in the ER (Kasinathan *et al.*, 1990; McIlhinney, 1989; Schmidt and Burns, 1989; Berger and Schmidt, 1984a), in Golgi (Gutierrez and Magee, 1991), and plasma membrane (Das *et al.*, 1997; Dunphy *et al.*, 1996). Das *et al.*, (1997) has successfully purified a 70kDa acyltransferase from human erythrocytes specific for LCACoA esters. Accordingly, Dunphy *et al.* (1996) and Berthiaume and Resh (1995) both described a protein acyltransferase activity from bovine brain plasma membranes with a modest preference for palmitoyl-CoA over other long-chain fatty acyl-CoA esters like myristoyl-CoA, stearoyl-CoA and arachidonoyl-CoA. Likewise, myristoyl-, palmitoyl-, and oleoyl-CoA are better substrates than short-chain acyl-CoA esters for *in vitro* acylation of myelin proteolipid protein (Bizzozero *et al.*, 1987b). However, the relatively unrestricted use of fatty acyl-CoA esters as substrates by these enzymes may result in the

observed heterogeneity of thioacylated proteins based upon their lipid content. Palmitic, stearic, and oleic acid are the major acyl-chains found in brain myelin proteolipid protein (Bizzozero *et al.*, 1987b), P-selectin is acylated with either palmitate or stearic acid (Fujimoto *et al.*, 1993), the transferrin receptor can be posttranslationally modified by myristate, palmitate, or stearate (Nadler *et al.*, 1994), while the α -subunit of heterotrimeric G-proteins can be covalently modified by arachidonate in human platelets (Hallak *et al.*, 1994). Due to the lack of any pronounced substrate specificity among the acyltransferases, it is notable that very-long chain fatty acids are bound to myelin proteolipid protein in patients suffering from adrenoleukodystrophy (Bizzozero *et al.*, 1991), indicating that the pool of acyl-CoA esters to some extent determines the fatty acid composition of an acylated protein.

The precise intracellular localization seems to depend on the specific protein. Fatty acylation of several polytopic membrane proteins such as the nicotinic acetylcholine receptor, the α -subunit of the voltage-gated Na-channel, G-protein coupled receptors (α_1 -, α_2 -, β_1 -, and β_2 adrenergic receptors, the serotonin 5HT receptor, and dopamine receptors (DID2L), and rhodopsin) as well as a number of hydrophilic proteins including growth-cone protein 43 (GAP-43), p21^{H/N/K-ras}, small ras-like GTP binding proteins (rab), α -subunits of heterotrimeric G-proteins, endothelial nitrogen oxide synthase (eNOS), and non-receptor tyrosine kinases belonging to the Src family like Fyn, Hck, and Lck all appear to take place at the plasma membrane (Wedegaertner *et al.*, 1995; Hancock *et al.*, 1989; Olson and Spizz, 1986). Acylation of bitopic membrane proteins like the myelin PO glycoreceptor, the neural cell adhesion molecules NCAM, the transferrin receptor, and the cation-dependent mannose 6-phosphate receptor occurs in the ER/Golgi apparatus (Dolci and Palade, 1985; Schmidt and Schlesinger, 1980).

The most remarkable difference between palmitoylation and other lipid modifications of proteins like N-myristoylation and prenylation is its dynamic nature. The half lives of the protein bound acyl moieties can range from minutes to days (Milligan *et al.*, 1995; Bizzozero and Good, 1991; Magee *et al.*, 1987) and acylation of a large number of proteins is insensitive to inhibition of protein synthesis (Magee *et al.*, 1987; Townsend *et al.*, 1982). This may indicate that acylation and deacylation serve to regulate the activity of proteins, rather than to define the function. Hence, it has been suggested that protein acylation is a *bona fide* regulatory mechanism similar to protein phosphorylation. Dynamic palmitoylation plays an important role in coupling of the human β_2 -adrenergic receptor to its G-protein (O'Dowd *et al.*, 1989), in cycling of heterotrimeric G-proteins between basal and agonist-activated state (reviewed in Mumby, 1997), and in regulation

of subunit interaction in receptors or ion channels (Olson *et al.*, 1984). The dynamic turnover of protein bound fatty acids also suggests that palmitoylated proteins are subject to constant deacylation. Since thioesters are highly susceptible to nucleophilic attack it is possible that the fatty acid could be transferred to other protein thiols or to abundant intracellular nucleophiles like glutathione. Transacylation between synthetic peptides can take place *in vitro* (Quesnel and Silvius, 1994). However, depalmitoylation of proteins appear to occur enzymatically (Bizzozero *et al.*, 1992; Berger and Schmidt, 1986). An acyltransferase capable of removing fatty acyl groups from Semliki Forest virus E1 and E2 glycoproteins was identified in microsomes from BHK cells (Berger and Schmidt, 1986). Another membrane bound acyltransferase has been identified in brain myelin that deacylates brain myelin proteolipid protein (Bizzozero *et al.*, 1992). More recently, a palmitoyl protein thioesterase (PPT) was purified from bovine brain cytosol and subsequently cloned and sequenced (Camp *et al.*, 1994; Camp and Hofmann, 1993). PPT deacylates p21^{H-ras} and the α -subunits of heterotrimeric G-proteins, but is also capable of hydrolyzing saturated as well as unsaturated LCACoA esters (Camp *et al.*, 1994). However, the lysosomal localization of PPT makes it questionable as to whether or not PPT is involved in deacylation of proteins at their subcellular localization. Nevertheless, the biological significance of PPT in protein deacylation is underlined by its ubiquitous tissue expression and by the fact that mutations in the gene encoding for PPT cause the autosomal recessive brain disorder, infantile neuronal ceroid lipofuscinosis (Hofmann *et al.*, 1997). Due to its apparent lysosomal localization, mutations in the PPT gene may lead to accumulation of acylated proteins in lysosomes, or to a more global defect in lysosomal function (Hofmann *et al.*, 1997). Supporting this proposal it was noted that acylated phospholipase A₂ and salivary mucosal glycoprotein are both more resistant to proteolysis than their deacylated forms (Diaz *et al.*, 1995; Slomiany *et al.*, 1988). Alternatively, mutations in the PPT could eventually lead to accumulation of acyl-CoA esters in the lysosome due to the inability to hydrolyze LCACoA esters. This in turn might inhibit some proteases like proline peptidase (Yamakawa *et al.*, 1990). Stable expression of PPT in Sf-9 cells decreased incorporation of palmitate into total cellular lipids indicating that PPT may play a more general role in cellular lipid homeostasis (Michel and Michel, 1997).

4.3. Palmitoylation and Membrane Fusion, Similarities Between Influenza Virus Hemagglutinin and SNAREs

Fusion between lipid bilayers is a central step in virus-cell interaction and in virus infectivity. Since Schmidt *et al.* (1979) first identified

palmitoylated proteins in Sindbis Virus infected chicken embryo cells, a growing number of viral proteins, including the influenza virus hemagglutinin, have been shown to acquire covalently bound fatty acids (Lambrecht and Schmidt, 1986; Schmidt and Lambrecht, 1985). Hemagglutinin and neuroamidase are the two major envelope spike glycoproteins of the influenza A virus. Despite the fact that hemagglutinin is very well characterized with regards to its structure, biosynthesis, and posttranslational modifications (Bullough *et al.*, 1994), the role of palmitoylation of hemagglutinin is only poorly understood. Hemagglutinin is synthesized as a 550 amino acid precursor but is subsequently cleaved into two subunits HA1 and HA2 which associates to form trimers (Klenk and Garten, 1994). HA1 forms the globular domain of the hemagglutinin spike and is responsible for binding to sialic acid-containing receptors on host cells, while HA2 is responsible for fusion activity. Under fusion-inducing conditions the pH decreases slightly which results in a conformational change of HA2 which exposes a fusion peptide that binds hydrophobically to the target membrane and the viral membrane (Chernomordik *et al.*, 1997). These interactions are believed to induce membrane fusion, a process that is sensitive to changes in the lipid environment and specific mutations in the fusion protein (Chernomordik *et al.*, 1997; Kemble *et al.*, 1994). HA2 contains three conserved cysteine residues in the cytoplasmic domain of the protein in close proximity to its transmembrane region. Each cysteine residue is palmitoylated through a thioester bond (Ponimaskin and Schmidt, 1998; Philipp *et al.*, 1995; Steinhauer *et al.*, 1991; Veit *et al.*, 1991; Naeve and Williams, 1990). Acylation of hemagglutinin does not affect biosynthesis, transport or receptor binding, and the reported effects on fusion are inconsistent. Using site-directed mutagenesis Naeve and Williams (1990) showed that modification of any of the three cysteines abolished its membrane fusion activity. This supports the observation that deacylation of hemagglutinin inhibited viral fusion activity (Lambrecht and Schmidt, 1986). Recently it was shown that fatty acylation is important in pore flickering, an early event in the fusion process (Melikyan *et al.*, 1997). Additionally, elimination of acylation sites in hemagglutinin prevents formation of some infectious virus subtypes (Zurcher *et al.*, 1994) whereas no effect was observed on other subtypes (Jin *et al.*, 1997, 1996). In some acylation mutants of hemagglutinin syncytia formation was suppressed but no effect was reported on membrane fusion and pore formation (Fisher *et al.*, 1998). However, in some studies acylation did not alter the properties of hemagglutinin to any extent (Naim *et al.*, 1992; Veit *et al.*, 1991). Despite the inconsistency in the reported effects of hemagglutinin acylation, it is interesting that of the known HA serotypes, the five that have been examined with respect to acylation

are all palmitoylated. Moreover, the two cysteine residues that are strictly conserved among the known influenza type A serotypes, are also found in the influenza type B virus. Hence, the authors proposed that fatty acylation of hemagglutinin contributes to the stability and anchoring of the protein in the membrane during the pH-induced conformational change of hemagglutinin prior to membrane fusion (Naeve and Williams, 1990).

It is interesting that v- and t-SNAREs share some of the same features as viral fusion proteins. Certain influenza strains can form extremely stable viral hairpins that are capable of fusing. In this case, the viral fusion protein is inserted into the target membrane and links the viral and the target membranes much in the same way v- and t-SNAREs do (Weber *et al.*, 1998). It has recently been shown that formation of a SNAREpin by interaction between cognate v- and t-SNAREs leads to spontaneous membrane fusion (Weber *et al.*, 1998). Hence, the authors suggested that viral fusion proteins can be considered as SNAREpins, where the membrane anchor of the viral fusion protein represents a v-SNARE, while the hydrophobic fusion peptide represents a t-SNARE that is inserted into the target membrane upon activation. Like many viral fusion proteins a number of SNAREs are also palmitoylated. These are the V-SNAREs synaptotagmin (Veit *et al.*, 1996) and the yeast VAMP/synaptobrevin homologues Snc1p and Snc2p (Couve *et al.*, 1995), and the t-SNARE SNAP-25 (Veit *et al.*, 1996). The role of fatty acylation is not known. However, one role might be to contribute to the stability of the fusion complex. As indicated by the observed effect positive effect of LCACoA esters on vesicle fusion which takes place prior to the formation of a NEM-resistant fusion intermediate, but after binding of NSF to the prefusion complex (Pfanner *et al.*, 1990). This and the fact that increasing concentrations of acyl-CoA esters can overcome limiting amounts of NSF implies that NSF may be important in acylation of a fusion component although NSF itself is not acylated (Pfanner *et al.*, 1990). As suggested by Pfanner *et al.* (1990) protein acylation could provide one fusion component with a hydrophobic lipid moiety that participates in perturbation of lipid bilayers, or functions as an anchor to promote stable assembly of the fusion machinery with or within the membrane. Consequently, deacylation may be important in disassembly and repeated use of the fusion machinery. Given the synergistic relationship between NSF and acyl-CoA and the fact that NSF is likely to be involved in regeneration of the fusion machinery rather than in the actual fusion event, it seems likely that acyl-CoA esters play a role in the dynamic regeneration of essential fusion components.

4.4. A Putative Link Between Coat Assembly, Phospholipases, Protein Kinases and Acyl-CoA Esters

As reviewed above, the specific step in vesicle formation and membrane fusion which requires LCACoA esters remains elusive. Even though protein acylation is likely to play an important role in the vesicle trafficking pathway, it may also be the case that localized acyl chain remodeling of membrane lipids is essential in the final pinching off and fusion process of membrane vesicles. Recently, several lines of evidence have linked enzymes that modify membrane lipids to membrane trafficking. Besides binding to Golgi and directing the translocation of coatamer onto the membrane, ARF has also been found to be an effective activator of phospholipase D (PLD) (Ktistakis *et al.*, 1996; Cockcroft *et al.*, 1994; Brown *et al.*, 1993). ARF-activated PLD hydrolyzes phosphatidylcholine to produce phosphatidic acid and choline. Stimulation by ARF is inhibited by brefeldin A (Ktistakis *et al.*, 1995), which prevents association of ARF to Golgi membranes and inhibits secretion. In Golgi preparations from brefeldin A-resistant cells containing tightly bound ARF, PLD activity is elevated and remains unaffected by brefeldin A treatment (Ktistakis *et al.*, 1995). The model predicts that activation of PLD by ARF stimulates hydrolysis of phosphatidylcholine which then leads to high local concentrations of phosphatidic acid. In terms of lipid dynamics, this could evoke a net increase in negative surface charges which could facilitate recruitment of coatamer and other essential vesicle components (Bi *et al.*, 1997). This is consistent with the finding that pure coatamer associates better with artificial lipid vesicles containing phosphatidic acid than control vesicles. Coatamer binding was further increased by inclusion of phosphatidylinositol (4,5)-biphosphate. Another consequence of phosphatidic acid formation could be an alteration in membrane curvature leading to initiation of budding, which further could be facilitated by phosphatidylinositol (4,5)-biphosphate (Ktistakis *et al.*, 1996).

In parallel to activation by ARF, PLD is also potently stimulated by phosphatidylinositol (4,5)-biphosphate (Singer *et al.*, 1997; Brown *et al.*, 1993) and protein kinase C (PKC) (Simon *et al.*, 1996a, b; Singer *et al.*, 1996; Conricode *et al.*, 1992). While the regulation by phosphatidylinositol (4,5)-biphosphate places PLD alongside the phosphoinositol signaling pathways (reviewed by Singer *et al.*, 1997), the role of PKC in membrane trafficking may be linked to LCACoA esters. Several forms of PKC are associated with Golgi membranes (Lehel *et al.*, 1995; Saito *et al.*, 1989), and participate in regulated (Buccione *et al.*, 1994) and constitutive exocytosis (De Matteis *et al.*, 1993), as well as endosomal receptor trafficking (Cardone *et al.*, 1994). Calphostin C, a PKC inhibitor that acts on the regulatory domain of the

enzyme, inhibits transport from the ER to Golgi and from Golgi to the plasma membrane (Simon *et al.*, 1996b; Fabbri *et al.*, 1994), while phorbol esters enhanced vesicle generation (Simon *et al.*, 1996b). In both cases, the effects appear to be independent of the kinase activity (Simon *et al.*, 1996b; Fabbri *et al.*, 1994). Partially purified PLD is activated by PKC (presumably mediated through the regulatory domain of PKC) (Singer *et al.*, 1996), leading to phosphatidic acid formation and coatamer recruitment. Membrane association of β -COP and ARF have been shown to be enhanced or inhibited by phorbol esters and calphostin C, respectively (De Matteis *et al.*, 1993). It is noteworthy that PKC inhibitors suppress vesicle scission, the same stage at which LCACoA esters are required, without affecting coat assembly, but were required prior to coat assembly to exert the effect. In the context of acyl-CoA regulation, it is interesting that LCACoA esters have been found to affect PKC activity in both stimulatory as well as inhibitory ways. Both palmitoyl-CoA and oleoyl-CoA (27 μ M) were found to enhance particulate PKC-activity in human skin fibroblasts in the presence of Ca^{2+} , phosphatidylserine and diacylglycerol (Nesher and Boneh, 1994), while partially purified cytosolic PKC activity was enhanced 60–70% by 13.5 μ M palmitoyl- or oleoyl-CoA in the absence of diacylglycerol (DAG). LCACoA esters (20–30 μ M) stimulate PKC activity purified from rat brain, with or without added DAG but in the presence of phosphatidylserine and Ca^{2+} , the effect was lower in the absence of DAG (Bronfman *et al.*, 1988). Whether the effect of acyl-CoA on PKC is direct due to binding of acyl-CoA to the enzyme or indirect through a stimulation of the synthesis of diacylglycerol (Brindley, 1984) is unknown. Active site acylation has been suggested to represent a novel mode of regulating enzyme activity (Berthiaume *et al.*, 1994). Recently, it was shown that palmitoylation of the regulatory subunit of rabbit brain PKC associates the enzyme with cellular membranes in a manner which is distinct from the nonpalmitoylated form of the enzyme (Ford *et al.*, 1998). Membrane partitioning may therefore represent a novel mechanism by which PKC regulates its membrane-associated targets.

5. ACYL-COA-DEPENDENT LIPID REMODELING IN VESICLE TRAFFICKING

Phospholipids have a cylindrical shape and form a firm and flat lipid bilayer, which interspersed with membrane proteins, form various cellular membrane compartments. In contrast to phospholipids, lysophospholipids have a relatively large hydrophilic head group and a small hydrophobic tail which form an inverted cone structure (Lawrence *et al.*, 1994; Grataroli *et*

al., 1991), capable of modulating the membrane curvature. Due to this structure, lysophospholipids are believed to exert a destabilizing effect on the membrane structure (Grataroli *et al.*, 1991). Accordingly, when added to fusing membranes, lysophospholipids inhibit membrane fusion (Chernomordik *et al.*, 1997). Prior to the pinching off process and during vesicle assembly, it is therefore reasonable to suggest that a membrane destabilizing event, such as accumulation of lysophospholipids, occurs. Phospholipase A activity (removal of one acyl-chain from 1,2-diacylphospholipids) has been localized in the Golgi apparatus (Moreau and Morré, 1991) indicative of lysophospholipid synthesis which may account for such an event. Addition of phospholipase A₂ increased transport of vesicles from ER to Golgi supports the notion of the importance of lipid remodeling in membrane trafficking (Slomiany *et al.*, 1992). However, in order to maintain the membrane integrity after fusion of the adjoining regions of the lipid bilayer within the coated vesicle, the bilayer structure must be restabilized. This might be achieved by the removal of lysophospholipids, accomplished by reacylation of lysophospholipids catalyzed by acyl-CoA:lysophospholipid acyltransferase present in the Golgi apparatus (Lawrence *et al.*, 1994). This is consistent with the *in vitro* observation that acyl-CoA esters are only required in the actual pinching off process of mature and fully assembled vesicles (Ostermann *et al.*, 1993).

5.1. Acyl-CoA and Vesicle Trafficking, Lessons from Yeast Mutants

A number of yeast mutants have been isolated carrying deficiencies in genes encoding for enzymes involved in fatty acid synthesis (reviewed in Schneider and Kohlwein, 1997). Surprisingly, these mutant strains have provided insight into the role of fatty acids in organelle inheritance (Stewart and Yaffe, 1991), structure of nuclear membranes (Schneider *et al.*, 1996) and nuclear division (Saitoh *et al.*, 1996). A role for fatty acids in organelle inheritance was suggested based on the isolation of a yeast mutant, *mdm2*, which exhibited defects in both mitochondrial morphology and inheritance. In *mdm2* strains the mitochondria are abnormally clustered and rounded in shape and defective in transmission to buds. Additionally, mitochondrial distribution within mating projections failed. The cells appear normal with respect to nuclear division and transmission. The *mdm2* mutation is an allele of the *OLE1* gene, encoding the d9-fatty acid desaturase (Stukey *et al.*, 1989). Oleic acid supplementation complemented the phenotype of *mdm2*, suggesting that unsaturated fatty acids are involved in movement of specific cellular organelles (Stewart and Yaffe, 1991; Berger and Yaffe, 1996).

During the course of identification of yeast mutants defective in mRNA transport Schneider *et al.* (1996) isolated one mutant, *mtr7-1/acc1-7-1*, with severe perturbations in the structure of the nuclear envelope and the ER. The *MTR7* gene was cloned from a yeast genomic library by complementation and found to be allelic to *ACC1/FAS3* encoding acetyl-coA carboxylase, which catalyzes the initial and rate limiting step in fatty acid synthesis. Surprisingly the mutant phenotype was not rescued by exogenous fatty acids indicating that long-chain fatty acid synthesis was not required for the function missing in the *mtr* mutant. A nonbiotinylatable mutant allele of *ACC1* did not complement the *acc1* null allele and synthesis of very-long chain fatty acids was significantly reduced. Therefore, the authors suggested that malonyl-CoA dependent elongation of long-chain fatty acids to very-long chain fatty acids is required for its essential function (Schneider *et al.*, 1996). Hence, it was proposed that very-long chain fatty acids containing lipids are required in stabilizing the curvature of the nuclear pore complex in the nuclear membrane. Very-long chain fatty acids ($>C_{20}$) did not rescue the mutant phenotype perhaps due to poor import into the cell.

Two mutants of the fission yeast *Schizosaccharomyces pombe* that are defective in coordination of nuclear division and cytokinesis have recently been characterized (Saitoh *et al.*, 1996). These two mutants, *cut6* and *lsd1*, exhibit severe defects in nuclear division resulting in daughter nuclei which differ dramatically in size. Fluorescent *in situ* hybridization shows that sister chromatids are separated in *lsd1* cells, but appear highly compacted in one of the two daughter nuclei. Electron microscopic analysis of *lsd1* cells also reveal an asymmetric nuclear elongation and an unequal separation of nonchromosomal nuclear structures. The electron-dense nuclear material are absent from the small nuclei and contain highly compacted chromatin. Both *cut6*⁺ and *lsd1*⁺ are essential for viability, and were cloned by complementation from a *Schizosaccharomyces pombe* genomic DNA library. They encode acetyl-coA carboxylase and the α -subunit of fatty acid synthetase, respectively. Whereas palmitate supplementation fully depressed the *lsd1* phenotype, the *cut6* mutant could not be rescued by palmitate, like the *mtr7* mutant in *Saccharomyces cerevisiae*.

In all three cases described above the mutant enzymes are inactivated at the non-permissive temperature, leading to a decrease in endogenous fatty acid synthesis. Thus, insufficiency of fatty acids and acyl-CoA esters may affect the properties of certain cellular membranes leading to the aberrant phenotype. As suggested by Schneider and Kohlwein (1997) the structural defect shared among these mutants may be linked to changes in membrane stabilization and in the membrane curvature. With respect to *mdm2*, it is likely that shortage of unsaturated fatty acids will lead to

alterations in mitochondrial cardiolipin, in which unsaturated fatty acids are very abundant. Cone-shaped lipids, like cardiolipin, induce a negative curvature and promote the formation of stalk-like structures that are intermediates in hemifusion (Markin *et al.*, 1984), in contrast to inverted cones, like lysophospholipids, that inhibit fusion when added to fusing membranes (Chernomordik *et al.*, 1997). Interestingly, oleic acid is also cone-shaped and has been found to stimulate membrane fusion (Chernomordik *et al.*, 1997).

6. ALLOSTERIC EFFECTS OF LONG-CHAIN ACYL-COA ON VESICLE TRAFFICKING

6.1. Acyl-CoA Regulation of Ion Fluxes

In synaptic vesicle fusion docked vesicles do not fuse with membranes until triggered by the influx of Ca^{++} . The current belief is that the v-SNARE synaptotagmin interacts with the t-SNARE SNAP-25, β -SNAP, and NSF and forms a Ca^{++} responsive fusion complex (Schiavo *et al.*, 1997; Südhof and Rizo, 1996; Schiavo *et al.*, 1995; Brose *et al.*, 1992). Syntaxin, synaptotagmin, synaptobrevin, and SNAP-25 interact with N-type Ca^{++} -channels (el Far *et al.*, 1995; Sheng *et al.*, 1994; O'Connor *et al.*, 1993). Thus, it is conceivable that regulation of Ca^{++} -channels would alter the activity of such fusion complexes. Accordingly, it is noticeable that LCACoA esters have been found to stimulate Ca^{2+} release by the ryanodine-sensitive Ca^{2+} release channel in longitudinal tubules and terminal cisternae of sarcoplasmic reticulum from rabbit skeletal muscle (Fulceri *et al.*, 1994). Additionally, LCACoA potentiates the effect of cyclic ADP-ribose on Ca^{2+} release by the ryanodine Ca^{2+} channel in sea urchin eggs (Chini and Dousa, 1996). Fatty acyl-CoA esters appear to interact directly with the ryanodine-sensitive Ca^{2+} -channels since both palmitoyl-CoA and the corresponding nonhydrolysable analogue, induced release of Ca^{2+} with an EC_{50} of 6 μM . Ruthenium red, a Ca^{2+} release channel blocker, completely prevented this effect. Furthermore, palmitoyl-CoA increased the affinity of ryanodine binding without changing the binding capacity (Fulceri *et al.*, 1994). Similar effects of LCACoA were observed on the ryanodine receptor from duckling sarcoplasmic reticulum, at concentrations below 20 μM (Dumonteil *et al.*, 1993, 1994).

However, a number of other studies suggest that LCACoA esters exert opposing effects on vesicle fusion and ion fluxes. In contrast to the stimulatory effect of palmitoyl-CoA on vesicular transport in the Golgi apparatus, low concentrations of LCACoA and CoA also inhibit GTP-dependent fusion of rat liver microsomal vesicles with IC_{50} -values ranging from

15–18 μM for C_{14} -, C_{16} -, and C_{18} -CoA esters whereas high acyl-CoA concentrations (50 μM) favored the formation of small microsomal vesicles (Comerford and Dawson, 1993). The authors suggested that GTP-induced membrane fusion in rat liver microsomes depends on a yet unknown acylation-deacylation mechanism required for complete vesicle sealing. However, this is unlikely since a non-hydrolyzable myristoyl-CoA analogue also blocks the GTP effect (Rys-Sikora *et al.*, 1994) indicating that the acyl-CoA effect is not caused by protein acylation but rather by an allosteric effect. Regulation of ion fluxes during fusion events may therefore play a key role in the recognition process prior to membrane fusion, ensuring that only vesicles with the same ion environment are allowed to fuse. The observed equivalence of palmitoyl- and myristoyl-CoA in inhibition of GTP-induced Ca^{2+} release supports this suggestion. Deeney *et al.* (1992) found that acyl-CoA esters (0.5 μM), but not the corresponding fatty acids, decreased the steady-state medium-free Ca^{2+} concentration in the clonal permeabilised pancreatic β -cells (HIT-cells) in a chain length- and concentration dependent manner. Mitochondrial inhibitors and PKC-depletion did not affect the ability of acyl-CoA to stimulate the Ca^{2+} uptake by the permeabilized cells, while thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} ATPase, blocked the acyl-CoA effect. Rys-Sikora *et al.* (1994) also observed that palmitoyl-CoA at low concentrations ($<1 \mu\text{M}$) induced uptake of Ca^{2+} in saponin-permeabilized DDT1MF-2 smooth muscle cells, whereas higher concentrations had an inhibitory effect on Ca^{2+} uptake. In liver, in contrast to muscle, low concentrations of acyl-CoA suppress GTP- and inositol-1,4,5-triphosphate induced Ca^{2+} -release from rat liver microsomal vesicles, and cause re-uptake of Ca^{2+} into and enlargement of the inositol-1,4,5-triphosphate sensitive compartment (Comerford and Dawson, 1993; Fulceri *et al.*, 1993). Larsson *et al.* (1996) demonstrated that the level of LCACoA increased more than 2-fold when clonal β -cells (HIT-T15) were incubated with palmitate for 18 hours. The same authors observed that LCACoA esters induced a rapid and slowly reversible opening of ATP-sensitive K^{+} -channels in a concentration dependent manner.

7. INTRACELLULAR ACYL-COA BINDING PROTEINS

7.1. Acyl-CoA Binding Protein

Termination of fatty acid synthesis in ruminant mammary gland is catalyzed by the medium-chain acyltransferase, an inherent part of the fatty acid synthase complex (Mikkelsen *et al.*, 1985). Termination results in

production of acyl-CoA esters, a process which is facilitated by an acyl-CoA consuming system such as bovine serum albumin, methylated cyclodextrins, or microsomal triacylglycerol synthesis (Hansen *et al.*, 1984a,b; Knudsen and Grunnet, 1982). Acyl-CoA binding protein (ACBP) was isolated as an impurity in a preparation of fatty acid binding protein on its ability to induce termination of medium-chain fatty acid synthesis by goat mammary gland fatty acid synthase (Mogensen *et al.*, 1987). ACBPs constitute a family of small homologous polypeptides which have been identified, purified or cloned from a plethora of eukaryotic species, ranging from yeast and plants to fish, birds, and mammals (reviewed in Færgeman and Knudsen, 1997). The broad range of distribution throughout the animal and plant kingdom and its high degree of similarity among the different tissues and species suggest that ACBP is a housekeeping protein. This was further supported by Mandrup *et al.* (1992) who demonstrated that the genomic gene of ACBP has all the characteristics of a housekeeping gene. ACBP is ubiquitously expressed in liver, adipose tissue, kidney, heart, brain, intestine, skeletal muscle, mammary gland (Mikkelsen and Knudsen, 1987) erythrocytes (Fyrst *et al.*, 1995) duodenum, testis, adrenal gland, ovary, lung and spleen (Bovolin *et al.*, 1990; Alho *et al.*, 1985). Recently, an ACBP isoform (endozepine-like peptide) was identified in mouse testes (Pusch *et al.*, 1996). The highest concentration of ACBP is found in liver where it is evenly distributed in all hepatocytes (Bovolin *et al.*, 1990). In other tissues ACBP is reported to be high in specialized cells like steroid producing cells of the adrenal cortex and testis, and in epithelial cells specialized in secretion and in water and electrolyte transport like the epithelial cells of kidney tubulus, the upper intestinal tract and the large bronchioles (Alho *et al.*, 1991; Bovolin *et al.*, 1990) clear cells of the human eccrine sweat glands, secretory cells of sebaceous glands, and in granular cells of epidermis (Alho *et al.*, 1993). ACBP is also highly expressed in the choroid plexus and circumventricular organs of the brain (Alho *et al.*, 1988; Shoyab *et al.*, 1986) which are specialized in controlling secretion and osmolarity of the cerebrospinal fluid. With regards to the role of acyl-CoA esters in membrane trafficking and secretion it is interesting that rat brain ACBP has been reported to be enriched in the synaptosomal fraction, apparently the synaptic vesicles (Ferrarese *et al.*, 1987). In *Drosophila melanogaster* ACBP has been found primarily expressed in tissues which were associated with high energy production or fat metabolism (Kolmer *et al.*, 1994).

ACBP binds medium- and LCACoA esters with very high affinity, with a preference for C₁₄-C₂₂ acyl-CoA esters (Færgeman *et al.*, 1996; Rosendal *et al.*, 1993). ACBP shows only low affinity towards CoA ($K_D = 2\mu\text{M}$) and does not bind fatty acids, acyl carnitines, cholesterol and a number of nucleotides (Rosendal *et al.*, 1993). Employing isothermal

microcalorimetry, the K_D values for palmitoyl-CoA binding to bovine, yeast, and rat ACBP were determined to 2.0, 2.3, and 8.9 nM, respectively (Fulceri *et al.*, 1997). Results from a number of *in vitro* and *in vivo* experimental results strongly indicate that ACBP is able to act as an intracellular acyl-CoA transporter and pool former. *In vitro*, ACBP has a strong attenuating effect on the inhibition of acetyl-coA carboxylase and on the mitochondrial adenine nucleotide translocase by LCACoA (Rasmussen *et al.*, 1993), it readily prevents membrane binding and insertion and protects acyl-CoA against hydrolysis by microsomal hydrolases and stimulates the mitochondrial LCACoA synthetase (Rasmussen *et al.*, 1993). Overexpressing of either bovine or yeast ACBP in *Saccharomyces cerevisiae* led to an increased intracellular acyl-CoA level indicating that ACBP is able to act as an acyl-CoA pool former *in vivo* (Knudsen *et al.*, 1994; Mandrup *et al.*, 1993). ACBP is able to desorb acyl-CoA esters immobilized in multilamellar liposomes on a nitrocellulose membrane, and transport and donate these to mitochondrial β -oxidation and to microsomal glycerolipid synthesis (Rasmussen *et al.*, 1994). Compelling evidence that ACBP participates in acyl-CoA transport *in vivo* has been obtained from yeast. Disruption of the ACBP-gene in *Saccharomyces cerevisiae* results in a dramatic perturbation of the acyl-CoA level and composition (Scherling *et al.*, 1996). The level of total acyl-CoA and stearoyl-CoA was increased 2.5 and 7.0 fold respectively. Despite of that, the δ -9 desaturase mRNA level in the ACBP knock-out strain was increased 3-fold, no change in the synthesis of monounsaturated fatty acids or the overall fatty acid composition in the knock out strain could be observed. These results strongly suggest that the increased stearoyl-CoA pool in the ACBP knock-out strain was not available for the δ -9 desaturase and that the ACBP knock out strain has a defect in intracellular acyl-CoA transport.

7.2. Fatty Acid Binding Protein and Sterol Carrier Protein-2 in Acyl-CoA Metabolism

Ever since it was noted that fatty acid binding proteins (FABPs) are capable of binding acyl-CoA esters with appreciable affinity (Mishkin and Turcotte, 1974a; Ketterer *et al.*, 1976) FABPs have been suggested to play an important role in acyl-CoA metabolism. FABPs constitute a family of proteins with a molecular mass of 14–15 kDa, which are abundantly present in the cytoplasm of tissues that are involved in the uptake or utilization of fatty acids. All FABP types bind long-chain fatty acids with K_D -values ranging from 10 nM to 1.0 μ M. (Frolov *et al.*, 1997; Rolf *et al.*, 1995; Paulussen *et al.*, 1988; Burrier *et al.*, 1987; Ketterer *et al.*, 1976; Mishkin and Turcotte, 1974a). The binding stoichiometry is one mole ligand per mole protein

except for liver FABP which binds 2 moles of long-chain fatty acid or LCACoA per mole. Liver FABP appears to function as a general hydrophobic anionic ligand binding protein, whereas the other FABP types evidently are more specific and preferably bind long-chain fatty acids and acyl carnitine-esters. Employing fluorescence spectroscopy the affinity constants of rat L-FABP has recently been determined to 8 and 97nM and 10 and 180 nM for *cis*- and *trans*-parinaroyl-CoA, respectively (Frolov *et al.*, 1997). These affinity constants are at least one order of magnitude lower than values previously reported (Rolf *et al.*, 1995; Hubbel *et al.*, 1994; Paulussen and Veerkamp, 1990; Storch *et al.*, 1989). FABPs bind acyl carnitine esters with K_D values in the same order of magnitude as for fatty acids (Paulussen and Veerkamp, 1990). The observation that liver FABP binds acyl-CoA with an affinity, which is comparable to the affinity of ACBP, suggests that FABP may have an important role in acyl-CoA metabolism. FABPs have been shown to enhance the activity of several microsomal acyl-CoA utilizing enzymes involved in cholesterol-ester-, phospholipid- and triacylglycerol synthesis, i.e. acyl-CoA:glycerol-3-phosphate acyltransferase (Jolly *et al.*, 1997; Haq *et al.*, 1987; Burnett *et al.*, 1979; Mishkin and Turcotte, 1974b) diacylglycerol acyltransferase (Iritani *et al.*, 1980; O'Doherty and Kuksis, 1975) lysophosphatidic acid acyltransferase (Bordewick *et al.*, 1985), and acyl-CoA: cholesterol acyltransferase (Scallen *et al.*, 1985; Grinstead *et al.*, 1983). Rat liver FABP appears to have both stimulatory and inhibitory effects on both mitochondrial and microsomal acyl-CoA synthetases (Noy *et al.*, 1986; Burnett *et al.*, 1979; Ockner and Manning, 1976; Wu-Rideout *et al.*, 1976). Rat liver FABP is able to reverse the inhibitory effect of acyl-CoA on the mitochondrial adenine nucleotide translocase (Barbour and Chan, 1979). FABP has also been shown to reverse the inhibitory effect of palmitoyl-CoA on acetyl-coA carboxylase activity (Lunzer *et al.*, 1977). However, to what extent FABP is able to bind and transport LCACoA esters *in vivo* is not known. The inability of liver FABP to release product inhibition of the mitochondrial acyl-CoA synthetase *in vitro* (Rasmussen *et al.*, 1993) questions to what extent FABP is able to play a role in acyl-CoA metabolism *in vivo*.

Besides ACBP and FABP, sterol carrier protein-2 (SCP-2) has also been shown to bind LCACoA esters. Frolov *et al.* (1996) have recently reported that SCP-2, a protein primarily localized in mitochondria and peroxisomes, binds LCACoA with K_D values in the 2–4nM range, the same order of magnitude as ACBP. The function of SCP-2 has long been known to be associated with the metabolism of lipids, especially cholesterol (Wirtz, 1997). The physiological significance of the acyl-CoA binding ability of SCP-2 is unknown, but implies that SCP-2 is able to function in intracellular handling of LCACoA. While ACBP (and FABP) function in acyl-CoA

trafficking in the cytosol SCP-2 may serve this function within peroxisomes and mitochondria. In liver cytosol, the relative molar ratio of ACBP to SCP-2 has been reported to be approximately 5:1 (Gossett *et al.*, 1996), questioning that SCP-2 plays a major role in acyl-CoA metabolism in the cytosol. However, in mice lacking SCP-2 peroxisomal catabolism of branched fatty acyl-CoA esters is defective, whereas the liver content of triacylglycerol and cholesterol esters are reduced, suggesting that SCP-2 also is involved in intracellular transport and esterification of LCACoA (Seedorf *et al.*, 1998).

8. *IN VIVO* REGULATION OF LONG-CHAIN ACYL-COA ESTERS

In order to evaluate the physiological relevance of the regulatory effects of LCACoA esters, it is of great importance to consider the intracellular concentration of LCACoA esters. The total cellular concentration of LCACoA esters has been reported to be in the range of 5–160 μ M, depending on the tissue and its metabolic state (Fargeman and Knudsen, 1997 and references herein). The levels of acyl-CoA esters varies significantly in different metabolic conditions such as fasting (Sterchele *et al.*, 1994; Tubbs and Garland, 1964; Bortz and Lynen, 1963) diabetes (Tubbs and Garland, 1964) fat/glucose feeding (Tubbs and Garland, 1964) and ingestion of hypolipidemic drugs (Sterchele *et al.*, 1994; Berge *et al.*, 1983; Berge and Bakke, 1981).

8.1. Regulation of the Intracellular Acyl-CoA Concentration

The size of the intracellular pool of LCACoA esters is determined by the rates of fatty acyl-CoA synthesis and utilization. While acyl-CoA synthesis largely is determined by the rate of activation by acyl-CoA synthetases of either imported fatty acids, endogenously synthesized fatty acids, or fatty acids from lipolysis of cellular lipids, utilization is determined by the rate of degradation by β -oxidation, incorporation into cellular lipids, acyl-CoA hydrolysis or protein acylation. A number of observations suggest that fatty acid uptake is closely linked to fatty acid utilization. The current model suggests that fatty acid transport protein (FATP), fatty acid translocase (FAT) and the plasma membrane fatty acid binding protein (FABPpm) play important roles in fatty acid uptake (Schaffer and Lodish, 1994; Abumrad *et al.*, 1993; Sorrentino *et al.*, 1988). While FATP may work in conjunction with a fatty acyl-CoA synthetase (Martin *et al.*, 1997; Schaffer and Lodish, 1994), FAT may function with intracellular fatty acid binding

proteins (Spitsberg *et al.*, 1995; Van Nieuwenhoven *et al.*, 1995; Abumrad *et al.*, 1993; Vork *et al.*, 1993).

While the total cellular concentrations of fatty acyl-CoA esters have been determined (see Faergeman and Knudsen, 1997 for a review), the amount of fatty acyl-CoA available for utilization and regulatory purposes is only poorly understood. Due to the presence of a series of control points, the intracellular concentration of unbound LCACoA esters is kept extremely low. In this regulatory scheme, intracellular acyl-CoA binding proteins are assumed to play important roles. A number of reports suggests that the ratio of LCACoA to ACBP is close to one (Sterchele *et al.*, 1994; Rasmussen *et al.*, 1993). Based on the reported *in vitro* binding affinity and the cellular level of ACBP it was calculated that the unbound

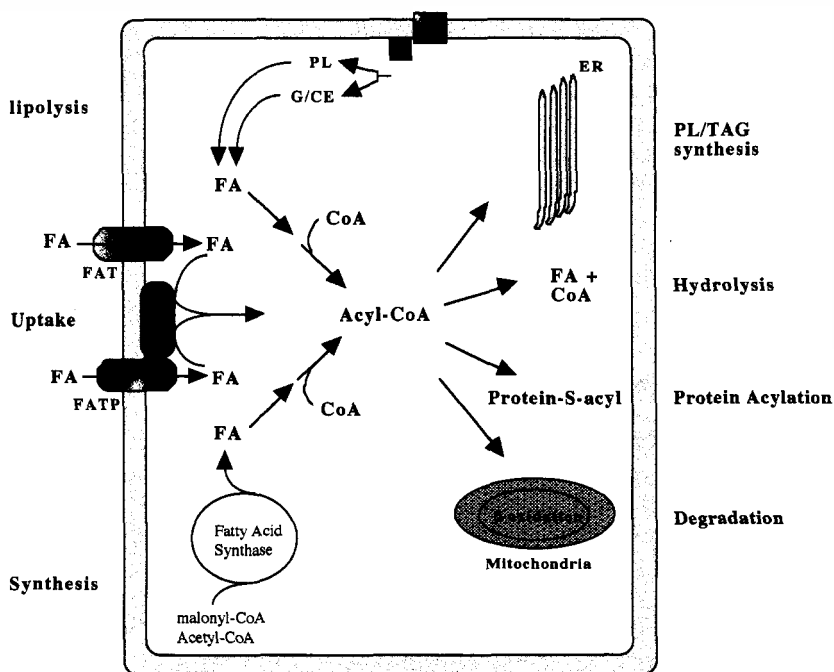


FIGURE 3. Regulation of the total acyl-CoA pool size. The size of the acyl-CoA pool is a balance between acyl-CoA synthesis and utilization. Acyl-CoA synthesis is determined by the rate of activation by acyl-CoA synthetases of either imported fatty acids, endogenously synthesized fatty acids, or fatty acids from lipolysis of cellular lipids. Utilization is determined by the rate of degradation by β -oxidation, incorporation into cellular lipids, acyl-CoA hydrolysis or protein acylation.

concentration of LCACoA would be in the low nM range when the acyl-CoA/ACBP ratio is below one. As this ratio exceeds one, FABP starts buffering and the level of unbound acyl-CoA was calculated to be less than $0.2\mu\text{M}$ (Faergeman and Knudsen, 1997). However, FABP may bind LCACoA esters with higher affinity than assumed in these calculations. Therefore, it is likely that the free concentration of LCACoA is even lower than described in Faergeman and Knudsen (1997).

LCACoA esters can also bind to a number of other proteins in the cell including the high affinity binding site on acyl-CoA synthetase and acyl-CoA utilizing enzymes. Webb *et al.* (1987) have identified an open reading frame in a bovine brain cDNA library which encodes a 533 amino acid protein containing an ACBP-like domain, termed bovine brain factor (bBF). Based on the amino acid sequence, it was suggested that bBF could be associated to either cell surface- or mitochondrial membrane (Todaro *et al.*, 1991). It is tempting to speculate that membrane-associated bBF binds fatty acyl-CoA with high affinity, and thus by competing with the cytosolic ACBP is able to create a local pool of membrane bound acyl-CoA esters. In addition, LCACoA esters readily partition into membranes. The association constant of palmitoyl-CoA for the interaction with phospholipid membranes has been determined to be approximately $1.5-5 \times 10^5 \text{ M}^{-1}$ (Requero *et al.*, 1995a; Peitzsch and McLaughlin, 1993). Applying these values to *in vivo* conditions the calculated free concentration of acyl-CoA in a liver cell in the absence of binding proteins would be about $1\mu\text{M}$. The *in vivo* cytosolic concentration of LCACoA in a liver cell can therefore be expected to be well below $0.2\mu\text{M}$ and never exceed $1\mu\text{M}$.

The size of the different acyl-CoA pools has also been postulated to be regulated by acyl-CoA hydrolases (Berge and Aarsland, 1985). Acyl-CoA hydrolases are found in most subcellular compartments and include short-, medium-, and LCACoA hydrolases (Lindquist *et al.*, 1998; Svensson *et al.*, 1998; Yamada *et al.*, 1998; Engberg *et al.*, 1997; Yamada *et al.*, 1997; Waku, 1992; Berge *et al.*, 1984; Berge, 1979; Berge and Farstad, 1979). Acyl-CoA hydrolases usually display K_m values ranging from $0.1-6\mu\text{M}$ for LCACoA esters (Yamada *et al.*, 1996; Broustas and Hajra, 1995; Berge, 1979). Some acyl-CoA hydrolases have been shown to respond to metabolic stresses, i.e. increasing by ingestion of hypolipidemic drugs (Lindquist *et al.*, 1998; Svensson *et al.*, 1998; Yamada *et al.*, 1998; Engberg *et al.*, 1997; Yamada *et al.*, 1997), which also results in an increase in the total acyl-CoA level in rat liver (Berge and Bakke, 1981). Moore *et al.* (1992) have shown that acyl-CoA hydrolase activity in rat adipocytes was decreased upon starvation, while the acyl-CoA level increased. Whether the intracellular acyl-CoA concentration is regulated by acyl-CoA hydrolase activity is not known. Nevertheless, it is very likely that acyl-CoA hydrolases could act as

“scavengers”, if the free LCACoA pool increases to μM concentrations. Furthermore, large fluctuations in the concentration of LCACoA esters in the cell will be prevented by the fact that the acyl-CoA synthetase is product inhibited by LCACoA esters, *i.e.* palmitoyl-CoA inhibits with a K_i of $4\mu\text{M}$ (Pande, 1973).

The most prominent intracellular fatty-acyl derivative is the acyl carnitine esters and evidences for a direct link between the LCACoA- and the long-chain acyl carnitine pool were provided by Arduini *et al.* (1997,1992, 1990). Long-chain carnitine acyltransferases (CPTs) constitute a family of enzymes present in the outer membrane of mitochondria, peroxisomes and endoplasmic reticulum (reviewed in Brady *et al.*, 1993; Ramsey and Arduini, 1993). The equilibrium constant for CPTI has been reported to be about one (Brady *et al.*, 1993). A flow of acyl chains between the acyl-CoA- and acyl carnitine pool is therefore likely, and the acyl carnitine pools will in this way act as buffer for activated acyl chains *in vivo*. The observations that acyl-CoA and acyl carnitine levels increase proportionally in both ischemic and hypoxic rat hearts (Whitmer *et al.*, 1978) and that the mitochondrial and cytosolic levels of acyl-CoA and acyl carnitine increase in parallel in rat heart (Idell-Wenger *et al.*, 1978) support this suggestion. Acylcarnitines may indirectly influence the level unbound concentration of LCACoA esters. If the total level of LCACoA exceeds the binding capacity of ACBP, LCACoA and acyl carnitine will start competing with each other for binding sites on FABP. Under these conditions, the calculated concentration of unbound LCACoA will not exceed $1\mu\text{M}$ (Faergeman and Knudsen, 1997). At this point the activity of the acyl-CoA hydrolases will be expected to be very high, preventing further increases in the intracellular concentration of LCACoA.

Compartmentation of LCACoA esters is another important unsolved issue. Only few attempts to estimate the intracellular distribution of LCACoA have been reported (Kobayashi and Fujisawa, 1994; Rasmussen *et al.*, 1993; Idell-Wenger *et al.*, 1978; Cannon *et al.*, 1977). The concentration of LCACoA in rat adipocyte mitochondria was determined to be $205\mu\text{M}$ in the fed state and increased upon 24 hours starvation to $258\mu\text{M}$. Using both control and mild ischemic rat hearts Idell-Wenger *et al.* (1978) found that 95% of total free and esterified cellular CoA was mitochondrial. Likewise, Kobayashi and Fujisawa (1994) found 92% of the cellular LCACoA to be located in the mitochondria from dog heart. From the original data by Rasmussen *et al.* (1993) it can be calculated that the LCACoA concentration in rat liver mitochondria is $230\mu\text{M}$, which constitutes only 15% of the total LCACoA. It has been suggested that 20–40% of the total acyl-CoA pool is cytosolic (Oram *et al.*, 1975). Deeney *et al.*, (1992) estimated the cytosolic LCACoA level in a clonal β -cell line to constitute

approximately 78% of the total LCACoA level, giving a cytosolic concentration of 90 μ M

9. REGULATION OF VESICLE TRAFFICKING *IN VIVO* BY LONG-CHAIN ACYL-COA ESTERS

Taking all of the above considerations into account we conclude that the intracellular free acyl-CoA concentration can not exceed 200 nM under normal physiological conditions. If the cytosolic ACBP/acyl-CoA ratio stays below 1 the free concentration will be in the 2–10 nM range. Fatty acid synthesis occurs despite that the K_i for inhibition of acetyl-coA carboxylase is 5.5nM (Ogiwara *et al.*, 1978) implying that the free concentration of liver cytosolic LCACoA is below 5.5nM during these conditions. We can not exclude that compartmentation of acyl-CoA esters can result in accumulation of LCACoA. The total level of acyl-CoA can increase to 1 mM in mitochondria (Idell-Wenger *et al.*, 1978). However, while ACBP and FABP act as buffer proteins in the cytosol, SCP-2 may prevent such an accumulation in mitochondria and peroxisomes. If the total free acyl-CoA concentration under normal physiological conditions is well below 200 nM and most likely below 10nM, the role of acyl-CoA as a physiological regulator of the cellular processes shown in table 1 will be expected to be limited to a few number of cellular processes. It can not be excluded that high local concentrations of LCACoA esters exist. The fact that a number of acyl-CoA synthetases localize to different cellular compartments lends credence to the belief of the existence of high local cellular concentrations of LCACoA esters. Whereas the stimulatory effect of palmitoyl-CoA on homotypic vacuole fusion in yeast which can be observed at approximately 100nM (Haas and Wickner, 1996), the effect on vesicle budding and fusion in the Golgi apparatus requires acyl-CoA in the low μ M range (Ostermann *et al.*, 1993; Pfanner *et al.*, 1990, 1989; Glick and Rothman, 1987). It has been demonstrated that acyl-CoA synthetase 1 is associated with GLUT4-containing vesicles (Sleeman *et al.*, 1998) which is likely to create a subcellular pool of LCACoA esters to support vesicle trafficking. It is of particular importance that no acyl-CoA binding proteins, to prevent acyl-CoA binding and accumulation in the Golgi membranes or yeast vacuoles, were present in the cell-free assay systems, which were employed to show the stimulatory effect of LCACoA esters on vesicle trafficking. However, the observations that LCACoA esters only are required in pinching off mature vesicles (Ostermann *et al.*, 1993) and in a specific step immediately prior to fusion (Pfanner *et al.*, 1990) support the notion of a specific function of LCACoA in vesicle trafficking. The effect appear to be mediated through

protein acylation or remodeling of membrane lipids since a non-hydrolyzable acyl-CoA analogue inhibits either membrane budding and fusion. Protein acylation is most likely catalyzed by a diverse set of protein acyltransferase *in vivo*, even though non-enzymatic protein acylation can occur *in vitro*. The biological significance of autoacylation is questionable since ACBP significantly reduces non-enzymatic acylation of some cysteine-containing synthetic peptides *in vitro* (Leventis *et al.*, 1997). At the present time the effect of specific acyl-CoA binding proteins on the function of LCACoA esters and protein acylation in vesicular trafficking is unknown.

Alternatively, acyl-CoA can be donated directly from a binding protein, ACBP, and perhaps FABP, to specific cellular processes. That this might occur is indicated by the observation that the acyl-CoA/ACBP complex at molar ratios below one can donate acyl-CoA for β -oxidation and glycerolipid synthesis (Rasmussen *et al.*, 1994) to the acyl-CoA:

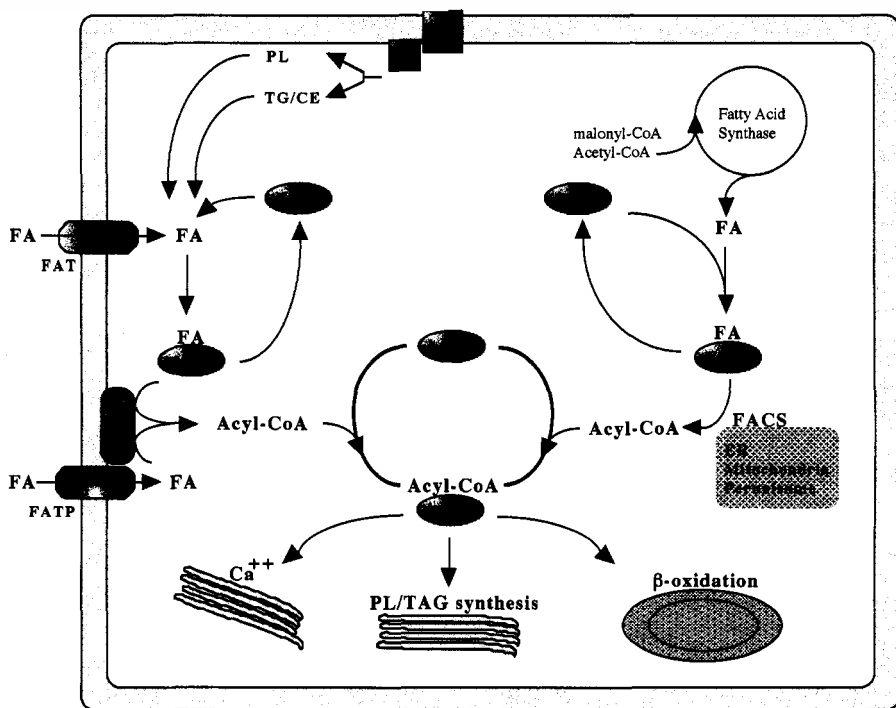


FIGURE 4. Role of ACBP in acyl-CoA pool formation. ACBP is able to create a pool of long-chain acyl-CoA esters which is available for specific purposes.

lysophospholipid acyltransferase in red blood cells (Fyrst *et al.*, 1995) and regulate sarcoplasmic ryanodine Ca^{2+} release channels (Fulceri *et al.*, 1997). It is therefore tempting to speculate that ACBP by binding LCACoA creates a pool of LCACoA available for specific purposes.

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Chapter 6

Brefeldin A Revealing the Fundamental Principles Governing Membrane Dynamics and Protein Transport

Catherine L. Jackson

1. INTRODUCTION

Brefeldin A (BFA) has enjoyed widespread use as an inhibitor of protein secretion in eukaryotic cells. BFA, first described in 1958 (Singleton *et al.*, 1958), is a heterocyclic lactone produced by certain fungi. In the mid-1980's, BFA was demonstrated to have dramatic effects on the structure and functioning of intracellular organelles, particularly the Golgi apparatus, in eukaryotic cells (Doms *et al.*, 1989; Hunziker *et al.*, 1992; Lippincott-Schwartz *et al.*, 1991; Lippincott-Schwartz *et al.*, 1989; Misumi *et al.*, 1986). This discovery led to intense interest in the drug both for understanding the mechanisms underlying the organization of intracellular compartments and as a tool to specifically inhibit the functioning of the secretory pathway

CATHERINE L. JACKSON Division of Biochemistry and Molecular Genetics, Department of Cellular and Molecular Biology, SBGM, Bat 142, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

in eukaryotic cells (Klausner *et al.*, 1992). In addition to its spectacular effects on the Golgi apparatus, BFA also affects the structure of the organelles of the endosomal system and lysosomes. However many transport pathways through these latter organelles are not completely blocked by the drug as is the case for transport of proteins out of the ER-Golgi system. Both *in vivo* and *in vitro*, BFA causes formation of unique tubular membrane structures. It has been proposed that BFA enhances directly the "fusogenicity" of intracellular membranes and so is a membrane fusogen. Indeed, BFA does promote promiscuous fusion of normally distinct membrane organelles *in vivo*, and fusion between distinct Golgi cisternae *in vitro*. In both cases, this unregulated fusion is mediated by the tubular structures induced by BFA treatment. However, recent studies support the idea that the membrane-fusion phenotypes induced by BFA are an indirect result of a misregulation of normal membrane dynamics. First, it was demonstrated that BFA specifically inhibits a Golgi-associated guanine-nucleotide exchange activity for the small GTP-binding protein ADP-ribosylation factor 1, or ARF1 (Donaldson *et al.*, 1992b; Helms and Rothman, 1992). Subsequently, Dascher and Balch demonstrated that expression of the dominant inactive form ARF1^{T31N} in cells resulted in a phenotype indistinguishable from that of cells treated with BFA (Dascher and Balch, 1994). The identification of the Sec7 domain ARF guanine nucleotide exchange factors (GEFs) and the demonstration that these GEFs are the major targets of BFA (at least in yeast) suggests that the effects of BFA are largely a result of inhibiting activation of ARF (Peyroche *et al.*, 1999). We also showed in this study that the mechanism of action of BFA is to stabilize an inactive ARF-GDP-Sec7 domain protein complex, thus mimicking the action of a dominant negative mutation.

In this review, I will discuss the early work describing the effects of BFA at both morphological and molecular levels. I will restrict most of my discussion to the ER-Golgi system, since the effects of BFA have been the most thoroughly studied at this level of the secretory pathway. Next, I will introduce the family of Sec7 domain ARF exchange factors, and discuss the studies showing that they are major targets of BFA both *in vitro* and *in vivo*. Finally, I will discuss the implications of the recently-elucidated mechanism of action of BFA on Sec7 domain ARF exchange factors in transport through the ER-Golgi system. However, before getting to all of this, I will first discuss the popular models for trafficking through the ER-Golgi system, and will introduce an alternative concept that has received little attention but which provides the best framework for understanding the diverse and variable effects of BFA in different cell types. Once again, I use the ER-Golgi system as an example, but the general conclusions can be applied to all intracellular trafficking pathways.

2. THE MORPHOLOGICAL BASIS OF TRANSPORT IN THE ER-GOLGI SYSTEM

2.1. The Classic Models: Anterograde Vesicular Transport and Cisternal Maturation

There are currently two models for the mechanism of transport from the ER through the Golgi apparatus that have gained widespread acceptance: the anterograde vesicular transport model and the cisternal maturation model (Farquhar, 1985; Glick and Malhotra, 1998). Both models are based on the vesicular transport paradigm, which has dominated thinking in the field since it was proposed by Palade and colleagues in the 1960's (Palade, 1975). This paradigm, which has provided the morphological/structural basis for our concepts of protein and membrane trafficking, postulates that vesicles bud from a donor compartment, then are targeted to their acceptor compartment where they dock and fuse, releasing their contents. In this model, the Golgi is divided into distinct compartments with characteristic resident glycosylation enzymes: the intermediate compartment (ERGIC), also known as VTCs (for vesicular-tubular clusters), the cis-, medial- and trans-Golgi cisternae, and the *trans* Golgi Network (TGN) (Farquhar and Palade, 1998). Forward-moving transport vesicles are postulated to carry anterograde-bound cargo in the forward direction from the ER to the first Golgi compartment and from cisterna to cisterna of the Golgi apparatus. This anterograde vesicular transport model makes several predictions, which have not been borne out by recent molecular data. First, the localization of Golgi enzymes is quite variable in different cell types, and enzymes are not restricted to single cisternae but are distributed in a gradient across the Golgi apparatus (Nilsson *et al.*, 1993; Rabouille *et al.*, 1995; Velasco *et al.*, 1993). Second, transport using the *in vitro* intra-Golgi transport assay developed by Rothman and colleagues was found to occur as efficiently in the absence of COPI vesicles as in their presence (Elazar *et al.*, 1994; Taylor *et al.*, 1994). Third, glycosylation enzymes, rather than anterograde cargo, are concentrated in COPI vesicles (Love *et al.*, 1998). Fourth, the SNARE hypothesis for vesicle fusion with the acceptor compartment predicts that a separate t-SNARE molecule is required for each vesicle fusion step, but in yeast there is only one Golgi t-SNARE (Pelham, 1998). This new data has encouraged the adoption of a new model, cisternal maturation, which again at its base rests on the idea of vesicular transport. The new model postulates that vesicles carry glycosylation enzymes and transport machinery to be recycled from a later compartment back to an earlier compartment, thus leading to maturation of each compartment (Glick and Malhotra, 1998; Pelham, 1998). This new model, in which the

“direction” that vesicles travel has been reversed does lead to a profound change in the concepts of membrane dynamics through the ER-Golgi system. The anterograde vesicular transport model postulates pre-existing compartments that receive and then emit transport vesicles that carry anterograde-bound cargo. This is conceptually the easiest model to visualize, since we can think of the secretory pathway as a series of discrete boxes with defined transport carriers moving from one to the next. In the maturation model, distinct Golgi compartments do not exist as stable entities in themselves, but rather undergo continual transformation as resident enzymes are removed and taken backwards to a previous compartment. B. Glick and colleagues developed a mathematical model that describes how Golgi enzymes could be concentrated in different regions of the Golgi stack in such a maturation model (Glick *et al.*, 1997). The central concept is a difference in affinity of different resident Golgi enzymes for the retrograde transport machinery or more specifically, for the COPI coat. This model accommodates recent data (using the *in vitro* intra-Golgi transport assay) showing that Golgi-derived vesicles which co-fractionate with COPI contain Golgi glycosylation enzymes rather than anterograde cargo (Love *et al.*, 1998). A variation of this model is that instead of carriers moving backwards from saccule to saccule in a stepwise manner, there is one single retrograde pathway from the *trans* Golgi/TGN to the *cis* side of the Golgi apparatus (which would explain the need for only one t-SNARE in the Golgi apparatus). However, the original mathematical model as described requires stepwise retrograde transport events to maintain a distinct distribution of enzymes (Glick *et al.*, 1997).

2.2. The Three-Dimensional Structure of Intracellular Organelles and the Concept of Membrane Transformation

Beginning in 1974, Alain Rambourg, Yves Clermont and their collaborators developed novel electron microscopy techniques that combined the use of selective impregnation methods of diverse membrane systems, observation with the aid of stereoscopic techniques, and sections of different thicknesses. These novel techniques permitted the visualisation of the three-dimensional structure of cellular organelles (Rambourg and Clermont, 1990). A particularly useful impregnation method used by Rambourg, Clermont and colleagues was that of Karnovsky, which selectively contrasts all intracellular membranes and hence allows visualisation of the entire ER-Golgi system in one preparation. Rambourg and Clermont were able to demonstrate in a definitive manner that the Golgi apparatus of mammalian cells does not consist of independent structures (stacks of saccules) but rather is one continuous entity with the appearance of a continuous ribbon

(Rambourg and Clermont, 1990). Throughout the length of the Golgi ribbon, poorly fenestrated compact zones (saccular zones) are interconnected by highly fenestrated if not exclusively tubular zones (intersaccular zones). Following a *cis-trans* axis, the compact zones can be subdivided into three compartments. The *cis* compartment consists of a system of closely anastomosed tubules which form a network of regular polygonal mesh. Due to its position, Rambourg and Clermont named this structure the "*cis* tubular network". The medial compartment consists of flattened elements (saccules) superposed, poorly fenestrated, and strictly parallel with each other. The *trans* compartment is formed by several superposed sacculo-tubular elements for which the spatial configuration is notably different than the elements of the underlying medial compartments. They present one poorly fenestrated saccular portion which is strictly parallel to the saccular portion of the underlying elements. This saccular portion is continuous at its periphery with a tubular portion (the *trans* tubular network) which does not enter into contact with another saccular zone but curves away from the Golgi ribbon into the cytoplasm surrounding the *trans* region of the Golgi apparatus. The rupture of these *trans* tubular networks liberates secretory granules and numerous residual vesicular-tubular structures into the cytoplasm.

In secretory cells, the secretory material, first uniformly distributed in the lumen of the Golgi elements, starts to accumulate in dilations located at apparently random locations of a saccular element of the compact zone situated at any level along the *cis-trans* axis of the Golgi ribbon (although most commonly in a saccule of the *trans* compartment). The drainage of the secretory material towards these dilations is accompanied by a progressive perforation and tubulation of the large saccular portions of the *trans* element. The secretory contents of the originally saccular elements are drained into the dilations during a progressive tubulation of the fenestrated saccule. Hence segregation of the secretory material (which results in formation of a progranule from the original dilation) is tightly coupled to membrane transformation (from saccular to fenestrated to tubular). When the tubular elements connecting the dilations/progranules reach the point of being devoid of secretory content, they fragment to liberate the pro-secretory granules at the *trans* face of the Golgi apparatus. This tubulation of the saccular portions which accompanies the segregation and maturation of the secretory material, and the subsequent rupture of the tubular systems at the *trans* face of the Golgi apparatus, are the manifestations of an intense level of protein and lipid sorting activity at this level of the organelle (Rambourg *et al.*, 1987).

Studies on the structural modulations of the Golgi apparatus of secreting cells under conditions of physiological stimulation and inhibition of

secretion (in prolactin cells of the pituitary gland of lactating rats or acinar cells of the mammary gland of such animals) have further demonstrated that the membranes of the Golgi apparatus are the object of a permanent reorganization, in this case at the *cis* face of the organelle (Clermont *et al.*, 1993; Rambourg *et al.*, 1993a). Moreover, these studies demonstrate that the Golgi apparatus arises as a result of continual renewal at the *cis* side. The data suggest that the *cis* tubular network progressively constructs itself, apparently as a result of the homotypic fusion of vesicles derived from the endoplasmic reticulum, and that this type of vesicle is likely not involved in intra-Golgi transport. Hence the concept of vesiculation is very important, but for reasons other than what the idea of shuttling of vesicles between compartments would suggest: it provides a discontinuity and perhaps even directly triggers the events that allow the development of the Golgi apparatus as a distinct organelle. These studies reinforce the concept that within the Golgi apparatus, transport is accompanied by a mechanism of membrane transformation and maturation: tubulation → fenestration → saccularization. Although not yet defined at the molecular level, this series of membrane transformations (and the reverse series) appears to be a fundamental characteristic of membrane-mediated transport events.

2.3. Regulated Forward Membrane Flux as the Driving Force for Anterograde Transport in the Exocytic Pathway

The central concept of membrane dynamics developed by Rambourg and Clermont rests on the demonstration of an anatomically continuous membrane flux within the Golgi apparatus. The development of this idea was made possible through the use of thermosensitive (*ts*) secretory mutants of the yeast *Saccharomyces cerevisiae*. In *ts* mutants, the product of the mutant gene is specifically inactivated at the restrictive temperature (37°C) leading to arrest of growth and manifestation of the mutant phenotype. At the permissive temperature (20°C) the mutant gene product is functional and so growth and secretion are normal.

Contrary to what is observed in mammalian cells, the Golgi apparatus of the wild type strain of the yeast *S. cerevisiae* comprises approximately thirty independent units distributed seemingly randomly throughout the cytoplasm. Each unit possesses a structure that is exclusively tubular and in the form of small tubular networks of polygonal mesh. There are at least two reasons for the dispersal of Golgi elements in *S. cerevisiae*, as opposed to mammalian cells (or even other yeasts such as *Pichia pastoris*). First, *S. cerevisiae* lacks the microtubule-dependent system that carries ER-Golgi transport vesicles and tubular elements (VTCs) to the pericentriolar region (Huffaker *et al.*, 1988). Second, there are more ER exit sites present in

S. cerevisiae compared to other organisms such as *Pichia pastoris*, since the former seems to lack a system that assembles exit sites together at the level of the ER (Rossanese *et al.*, 1999). However, despite these differences, the basic process of secretion is highly conserved between *S. cerevisiae* and higher eukaryotic cells (Kaiser and Huffaker, 1992).

The *Sec7-1* mutant was isolated in the original selection for secretory pathway mutants (*sec* mutants) in *S. cerevisiae* (Novick *et al.*, 1981; Novick *et al.*, 1980). In *Sec7-1* cells maintained at restrictive temperature, there is a block in the secretory pathway at the level of exit from the Golgi apparatus (Franzusoff and Schekman, 1989; Julius *et al.*, 1984; Nishikawa *et al.*, 1990; Novick *et al.*, 1981; Stevens *et al.*, 1982). Rambourg, Clermont, and Képès carried out a detailed study of the evolution of Golgi elements in the *Sec7-1* mutant held at restrictive temperature (Rambourg *et al.*, 1993b). Cells were prepared using the Karnovsky procedure consisting of impregnation with a 1 : 1 mixture of osmium tetroxide and potassium ferrocyanide. This technique allows visualization of glycosylated membrane proteins, with a gradient of coloration from the ER (faintly stained) to the late Golgi/plasma membrane (darkly stained). The stereoscopic techniques based on thick section electron microscopy that had been developed by Rambourg and Clermont were adapted to the yeast system to examine the three-dimensional structure of intracellular organelles. Over a time course of incubation of the *Sec7-1* mutant at 37°C, the Golgi tubular networks were progressively transformed into stacks of saccules whose superposed elements were interconnected along a *cis-trans* axis to form a membrane structure that is anatomically continuous. This process can be divided into several steps. First, after only seven minutes at 37°C, one or more of the numerous Golgi tubular elements became more extensive, with tubules accumulating in a network with the same type of three-dimensional organization as in the wild type situation. These networks were intensely stained, correlating well with the fact that transport of secretory proteins is blocked at the exit of the Golgi, after the Golgi glycosylation enzymes have acted. Very few ER-Golgi vesicles were seen at this time point, indicating that as in the wild type, they continue to be consumed rapidly at the *cis* side of the Golgi. After 15 minutes at restrictive temperature, the large, darkly stained tubular networks had become aligned in a parallel fashion. Some cells had accumulated highly fenestrated cisternae, suggesting that the tubular elements, once aligned in parallel, had begun to undergo filling in of the tubular mesh to form fenestrated saccules. After 30–60 minutes, only a few Golgi elements per cell were seen, and all were composed of five to eight stacked, poorly fenestrated saccules. Hence the process of membrane transformation from tubulation through fenestration to poorly fenestrated saccules had gone essentially to completion. Interestingly, even after longer periods of incubation at the nonpermissive

temperature (up to two hours), the number of saccules did not exceed a given number. This observation suggests that there is a regulatory mechanism in action that prevents unlimited increase in the number of Golgi cisternae. The process of anterograde membrane flux appeared to continue even after two hours at restrictive temperature, but instead of increasing the size of the Golgi apparatus, there was an accumulation of ER membranes. That these structures which accumulate in *Sec7-1* mutants are indeed bone fide Golgi elements is supported by the fact that known Golgi-localized proteins such as Ypt1p (Segev *et al.*, 1988), Rer1p (Sato *et al.*, 1995), Vps1 (Rothman *et al.*, 1990), and the TRAPP ER-Golgi vesicle-targeting complex (Sacher *et al.*, 1998), as well as proteins in transit through the secretory pathway such as DPAP B (Roberts *et al.*, 1989), are associated with them as determined by immunofluorescence analysis.

The most important concept demonstrated by the *Sec7-1* study is that anterograde transport of molecules through the Golgi apparatus is accomplished by forward membrane flux within an anatomically continuous membrane system (Rambourg *et al.*, 1993b). As mentioned above, transport of glycoproteins in *Sec7-1* mutants is blocked at the level of exit from the Golgi apparatus whereas earlier events of ER-through-Golgi transport continue. Morphologically, the result is an elaborate Golgi structure containing normally Golgi-localized proteins and secretory material that has been glycosylated by late Golgi enzymes. The traditional model of anterograde vesicular transport is hard to reconcile with these observations. The fact that these Golgi structures are continuous eliminates the classic idea that they are formed via vesicles shuttling between separate, unconnected compartments. The progressive evolution of tubular structures with time (which strikingly parallels the evolution of structures along the *cis-trans* axis of the mammalian Golgi) indicates that membrane transformation is the driving force and not vesicular transport. The fundamental principle in both cases is clearly seen: a tubular structure becomes organized into parallel arrays in which the holes of the mesh are filled in to form a fenestrated saccule, which then becomes progressively less fenestrated and more saccular by continued "filling in of holes". At first glance, it may seem that the cisternal maturation model can explain these observations, but upon close examination, it is clear that this model as well is not adequate. Cisternal maturation postulates a progressive transformation of one saccule into another by retrograde movement of material from a later compartment to an earlier compartment; in this model anterograde transport occurs by default. This model would predict that if transport was blocked at exit from the Golgi, but retrograde movement continued, all of the Golgi should accumulate as the first Golgi compartment. Given the biochemical data indicating processing by late Golgi enzymes in the *Sec7-1* mutant and

morphological data indicating that the tubular network resembles mid-Golgi saccules and not the ER-Golgi intermediate compartment (consisting of vesicular tubular clusters or VTCs), this prediction is not supported by the data. It could be that in addition to exit from the Golgi, retrograde transport in the *Sec7-1* mutant is blocked as well. In this case, the cisternal maturation model would predict that the system would not change upon incubation at the non-permissive temperature; this is obviously not the case. Hence it is impossible to explain the *Sec7-1* phenotype using a retrograde transport model such as cisternal maturation where anterograde transport occurs by default.

It is remarkable that a simple micro-organism in which an elaborate Golgi apparatus does not normally exist has the necessary structural and regulatory information to form such a structure. One might have imagined that such a feat would require proteins that are not conserved between yeast and higher eukaryotic cells (of which many examples exist). The *Sec7-1* study suggests that one of the main reasons that *S. cerevisiae* has such a poorly developed Golgi is that transport occurs too fast to allow accumulation of elaborate structures. Indeed, it has been shown that yeast has the capacity to transport at least one glycoprotein, HSP150, from the ER to the Golgi apparatus with a half-time of at most two minutes (Gaynor *et al.*, 1998a). HSP150 is a highly O-glycosylated protein, and in this very short transit through the secretory pathway receives multiple, elaborate carbohydrate chains that account for three times the molecular mass contributed by its polypeptide backbone (Lupashin *et al.*, 1992; Russo *et al.*, 1992). The *Sec7-1* experiment demonstrates that when flow is blocked at a specific point (exit from the Golgi), a membrane structure accumulates "behind" this block point. This membrane accumulation takes place in a regulated manner, with characteristics of Golgi formation in higher eukaryotes. The progressive organization of a tubular membrane network into parallel arrays and their transformation from tubules into saccules by progressive "filling in of holes" parallels exactly the membrane transformation events that transform the *cis* tubular element into Golgi saccular elements (compact zones). This remarkable correlation strongly supports the idea that the Golgi apparatus arises as a result of the unfolding of a regulatory program. The process does not normally take place in *S. cerevisiae* because these structural events require a slowing down of the membrane flow before they can be manifest. Another level of regulation that seems to be operating in *S. cerevisiae* is the limitation of the number of saccules that accumulate in a given Golgi stack: the most reported for the *Sec7-1* mutant is eleven. The nature of this regulation is completely unexplored in any system. An exciting consequence of these observations is that the molecular components responsible for the fundamental membrane transformation

events governing forward membrane flux (at both structural and regulatory levels) are conserved from yeast to mammalian cells. Hence the powerful genetic approaches available in *S. cerevisiae* can be used to identify these molecular components and to elucidate the mechanisms governing membrane dynamics in the ER-Golgi system. In conclusion, the Rambourg/Clermont view is that compartments arise as a result of the execution of a regulatory/developmental program based on a continual process of membrane flux, rather than being pre-existing stations into and out of which proteins and lipids pass.

3. MORPHOLOGICAL AND BIOCHEMICAL EFFECTS OF BFA

3.1. Early Studies of the Effects of BFA on the Secretory Pathway in Mammalian Cells

BFA was shown in two early studies to specifically inhibit secretion, without affecting protein synthesis or other cellular processes (Misumi *et al.*, 1986; Takatsuki and Tamura, 1985). In vesicular stomatitis virus-infected baby hamster kidney cells, BFA treatment inhibited cell surface expression of VSV-G protein and led to accumulation of VSV-G intracellularly (Takatsuki and Tamura, 1985). Interestingly, Takatsuki and Tamura noted that the VSV-G protein produced in the presence of BFA migrated faster than G protein from the untreated control, suggesting a defect in glycosylation of this protein when produced in the presence of BFA. In rat hepatocytes, BFA blocked secretion and late-Golgi processing of several marker proteins, and dilation of the ER was observed by electron microscopy in cells treated with a high concentration of BFA for several hours (Misumi *et al.*, 1986). In subsequent studies, the morphological effects of BFA on the Golgi apparatus were investigated, and quite surprisingly it was found that this organelle completely disassembled after BFA treatment (Doms *et al.*, 1989; Fujiwara *et al.*, 1988; Lippincott-Schwartz *et al.*, 1989). Even more striking was the observation that Golgi glycosylation enzymes could now be found in an active form in the ER. These studies demonstrated for the first time the existence of a Golgi-ER retrograde transport pathway. Such a pathway had been predicted from earlier work. The elegant study of Wieland *et al.* following bulk lipid flow from the ER through the Golgi apparatus to the cell surface estimated that half of the phospholipids in the ER are transported to the Golgi every ten minutes (Wieland *et al.*, 1987). This result led to the prediction of a recycling pathway at least for lipids from the Golgi to the ER to compensate for the massive forward flow (Wieland *et al.*, 1987). A second prediction of Golgi-ER transport came from studies of the mechanism of ER-localization

of ER-resident proteins possessing a KDEL/HDEL motif at their C-terminus (Munro and Pelham, 1987). A small proportion of such proteins (such as BiP) were found to come into contact with Golgi glycosyltransferases, presumably because they had escaped from the ER with forward flow and had been transported to the Golgi (Pelham, 1988). This observation led to the proposal of a recycling mechanism to capture the escaped ER resident proteins and bring them back to their original location (Pelham, 1988). The prediction was confirmed experimentally by the identification and characterization of a receptor for KDEL/HDEL proteins, ERD2 in yeast and its homologues in mammalian cells, which was shown indeed to cycle between the ER and the Golgi apparatus (Hsu *et al.*, 1992; Lewis and Pelham, 1990; Lewis and Pelham, 1992; Tang *et al.*, 1993).

Both immunofluorescence and ultrastructural analysis of the location of secretory proteins and resident Golgi enzymes in cells treated with BFA demonstrated that they were present in the ER (Doms *et al.*, 1989; Fujiwara *et al.*, 1988; Lippincott-Schwartz *et al.*, 1989). In one study, J. Lippincott-Schwartz and colleagues used the T cell antigen receptor (TCR), a multi-subunit complex which assembles in the ER and is transported via the Golgi apparatus either to the lysosome or to the cell surface, as a marker to study the effects of BFA in murine T cell hybridoma cells (which contain all seven subunits of the receptor) or murine thymoma cells (which lack two subunits and hence retain the incomplete complex in the ER) (Lippincott-Schwartz *et al.*, 1989). In the former cell line, BFA treatment led to retention of the TCR in the ER and its processing by *cis* and medial Golgi enzymes. To eliminate the possibility that this effect was due only to newly-synthesized glycosylation enzymes being prematurely activated in the ER, cells expressing the ER-retained partial TCR complex were treated with cycloheximide and then treated with BFA. Under these conditions, the TCR γ subunit was processed by Golgi glycosylation enzymes, indicating that the latter had undergone transport from the Golgi apparatus to the ER. This study also demonstrated directly that a Golgi resident protein (mannosidase 11) was transported from the Golgi to the ER in the presence of BFA (Lippincott-Schwartz *et al.*, 1989). The work of Doms *et al.* confirmed the results of this study, using tsO45-VSV G-infected CHO cells (Doms *et al.*, 1989). Both studies demonstrated that complete redistribution of the Golgi into the ER took place in 10–15 minutes, a remarkable result that demonstrated for the first time the dynamic nature of the Golgi apparatus.

3.2. Conflicting Reports: Does the Golgi Disappear or Not?

Although in the first systems examined, the Golgi apparatus disappeared completely upon BFA treatment and Golgi enzymes relocated to

the ER, subsequent studies in a variety of cell types indicated that this phenomenon was not always observed (Hurtley, 1992). In rat pancreatic exocrine cells, it was reported that clusters of vesicles, positive for α -mannosidase II and ERGIC58 by immunogold electron microscopic analysis, remained after 3 hours of treatment with BFA (Hendricks *et al.*, 1992a). These markers could not be detected on ER structures, indicating that in these cells, Golgi enzymes do not translocate into the ER in response to BFA. In murine erythroleukemia (MEL) cells treated with BFA (Ulmer and Palade, 1991), the Golgi apparatus became disorganized immediately after BFA treatment (five minutes), vesicles accumulated, and Golgi membranes were no longer apparent after 30–60 minutes in the presence of BFA. After prolonged exposure to BFA (three to six hours), ER-associated tubular networks increased in frequency, and there was an accumulation of tubular structures in a given network (in continuity with regular ER cisternae). These tubular networks represent a modified portion of the ER located near the Golgi apparatus and are the site from which virus budding occurs in untreated infected cells. After BFA treatment for three hours followed by removal of the drug, vesicles appeared to fuse together and Golgi stacks were reformed, starting as early as one minute after BFA wash-out (Ulmer and Palade, 1991). These two studies are representative of a number of reports of Golgi elements remaining after BFA treatment (other examples include De Lemos-Chiarandini *et al.*, 1992; Hidalgo *et al.*, 1992; Komhoff *et al.*, 1994; Pavelka and Ellinger, 1993). All of these studies have in common the feature that Golgi reorganization (often described as tubulation) takes place immediately after BFA treatment, but instead of resulting in fusion of the totality of the Golgi with the ER, some residual Golgi-like structures remain.

The most extreme example on the other end of the spectrum from complete disappearance of the Golgi apparatus is in the case of early rat spermatids. In these cells, BFA has little effect on the structure of the Golgi apparatus, but only appears to give rise to an accumulation of ER membranes (A. Rambourg and Y. Clermont, personal communication).

In subsequent studies it was demonstrated that even in cells for which Golgi glycosylation enzymes are completely relocated to the ER, the trafficking of the ERGIC resident protein ERGIC53/58 and of the KDEL receptor continue in the presence of BFA, and that the ERGIC compartment is still present (Lippincott-Schwartz *et al.*, 1990; Tang *et al.*, 1995). Sandvig *et al.* compared the effects of BFA on morphology of the ER-Golgi system of five different cell lines, and found that in all except MDCK and PtK2 cells (resistant to BFA at the ER-Golgi level, see below), there was an accumulation of tubular/vesicular structures in close association with the ER, perhaps corresponding to the ERGIC compartment or a modified version (Sandvig *et al.*, 1991). In influenza virus-infected cells treated with

BFA, influenza virus hemagglutinin (HA) monomers and trimers (which distribute into the ER and Golgi, respectively, in untreated cells) were found in distinct subcompartments of the ER. These subcompartments were in communication but had different fractionation properties (Russ *et al.*, 1991). BFA treatment leads to a loss of compartment identity, but the extent to which the ER and Golgi remain distinct, both biochemically and structurally, appears to vary in different systems.

Recent studies using GFP-labelled proteins to directly visualize the effects of BFA in living cells has led to a better understanding of the morphological effects of BFA on the ER-Golgi system (Presley *et al.*, 1998). Sciaky *et al.* showed that redistribution of Golgi membranes into the ER is a two-step process (Sciaky *et al.*, 1997). The Golgi apparatus first undergoes extensive tubulation (as was observed by classic immunofluorescence analysis), which can only be visualized in the presence of intact microtubules. Next, a very dramatic and rapid emptying of Golgi contents occurs over a period of only 15 to 30 seconds. The interpretation of these results is that when one Golgi tubule comes into contact with a region of the ER, a fusion event occurs which leads to "absorption" of the Golgi into the ER. Hence the membranes do not simply mix by a diffusion-mediated process alone, but fusion is driven by the fact that the Golgi membranes are in a higher energy state than those of the ER (Sciaky *et al.*, 1997).

3.3. The Effects of BFA in Yeast

The effects of BFA have been examined in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In *S. pombe* cells, BFA causes the Golgi apparatus to disappear, accumulation of ER membranes and a block in secretion, as in mammalian cells (Turi *et al.*, 1994). Attempts to identify the target of BFA using genetic approaches with this yeast were not successful, leading instead to the identification of multi-drug resistance factors (Nagao *et al.*, 1995; Turi and Rose, 1995).

The morphological effects of BFA treatment on *S. cerevisiae* cells are consistent with those observed in other organisms: disappearance of normal Golgi structures, the appearance of abnormal *cis*-Golgi-like elements in close association with the ER, and finally to an accumulation of ER membrane after prolonged periods in the presence of the drug (Rambourg *et al.*, 1995; Vogel *et al.*, 1993).

BFA was shown to block early stages of ER-Golgi transport in *S. cerevisiae*, but not later transport events (Graham *et al.*, 1993). For the vacuolar hydrolase carboxypeptidase Y (CPY), BFA blocked further transport of the ER precursor form immediately, but the Golgi precursor p2 was transported to the vacuole where it underwent proteolytic activation normally. For the secreted protein α -factor, the late-Golgi α 1,3-modified form was

secreted normally from cells, whereas the early-Golgi $\alpha 1,6$ -modified form was blocked from further transport (Graham *et al.*, 1993). Immediately after BFA treatment, none of the secreted protein invertase was blocked in its ER-modified form, but received *cis*-Golgi modifications indicating "transport" to an early Golgi compartment (Gaynor and Emr, 1997). This result indicates that BFA has the same type of cargo-selective defect as was seen for *Sec21-3*, defective in the gamma subunit of COPI (Gaynor and Emr, 1997). The localization of Kex2p was seemingly not affected by BFA treatment as determined by immunofluorescence microscopy (Graham *et al.*, 1993). After prolonged periods of BFA treatment, translocation of certain proteins (including Kar2p/BiP and the vacuolar hydrolase CPY) into the ER was inhibited (Graham *et al.*, 1993; Vogel *et al.*, 1993). In most mammalian systems examined there is at least partial redistribution of normally Golgi-resident enzymes into the ER, including cells in which Golgi vesicular structures remain. One notable exception is pancreatic acinar cells, as described above. *S. cerevisiae* provides a second example of the lack of transfer of Golgi enzymes into the bulk of the ER in response to BFA, as ER-resident proteins do not receive Golgi glycosyltransferase modifications after BFA treatment (Graham *et al.*, 1993).

3.4. The Effects of BFA on the Endocytic Pathway and Lysosomes: Fusion of Organelles within Systems and Traffic Jams

BFA was found to affect both organelle morphology and trafficking patterns within the TGN/endosome/lysosome system, although the effects were more subtle than in the ER-Golgi system (Hunziker *et al.*, 1992). It was demonstrated that the TGN did not follow the rest of the Golgi apparatus back to the ER in the presence of BFA, although its structure was modified (Chege and Pfeffer, 1990; Ladinsky and Howell, 1992). Further studies showed that the TGN extended long tubules that fused with the early/recycling endosome (Lippincott-Schwartz *et al.*, 1991; Reaves and Banting, 1992; Wood *et al.*, 1991). Tubule formation and fusion was dependent on intact microtubules, as for fusion of the Golgi apparatus with the ER. Endocytosis of material and its delivery to the early endosome was not in general inhibited, nor was recycling of material from this compartment back to the cell surface. Lysosomes were also affected morphologically by BFA in some cell types, with BFA causing this organelle to extend long tubular processes that did not fuse with other organelles but resulted in a tubular lysosomal structure (Lippincott-Schwartz *et al.*, 1991; Wood and Brown, 1992). However, in other systems (such as MDCK cells), no morphological effects on lysosomes were observed (Prydz *et al.*, 1992). Similarly, in a study of the effects of BFA on AtT20 and Hela cells, the entire early

endosomal system fused into one large tubular network, whereas the morphology of the late endosome/prelysosome was not affected (Tooze and Hollinshead, 1992). The tubular lysosomal structures induced by BFA treatment can be found in certain cell types naturally, suggesting that BFA is accentuating a normal process, or disrupting an equilibrium between tubular and vacuolar structures (Wood and Brown, 1992).

The specific post-Golgi trafficking pathways that are blocked by BFA seem to differ substantially in different cell types. For example, in pancreatic acinar cells (as in the yeast *S. cerevisiae*), post-Golgi secretion continued normally in the presence of BFA (Graham *et al.*, 1993; Hendricks *et al.*, 1992b). In BHK-21 cells, however, secretion of VSV-G from the TGN to the cell surface was blocked (Miller *et al.*, 1992). In general, transport from the Golgi/TGN to lysosomes continues in the presence of BFA (Strous *et al.*, 1993). Maturation of secretory granules in AtT-20 cells has both BFA sensitive and resistant steps (Fernandez *et al.*, 1997).

Surprisingly, it was found that two kidney epithelial cell lines, MDCK and PtK1, were completely resistant to the effects of BFA at the level of the Golgi apparatus, but that the TGN/endosome system was sensitive to BFA (Hunziker *et al.*, 1992; Ktistakis *et al.*, 1991). Transcytosis of certain markers from the basal to the apical surface was completely blocked by BFA in MDCK cells (Hunziker *et al.*, 1991; Low *et al.*, 1992), whereas other transcellular pathways were not affected or were even stimulated by BFA (Prydz *et al.*, 1992; Sandvig *et al.*, 1991). It is likely that BFA is disrupting specific sorting pathways in the TGN/endosomal system, and that the specific effect depends on the marker used (Apodaca *et al.*, 1993; Mundigl *et al.*, 1993; Orzech *et al.*, 1999).

3.5. A Model to Explain the Morphological Effects of BFA

The wide range of BFA effects on the ER-Golgi system is difficult to rationalize using the classic models for ER-through-Golgi transport. With the idea of regulated membrane flux as the driving force for transport, a coherent explanation emerges. Compartments are the result of regulatory elements holding up membrane flux at particular points. In different cell types, these regulators will “set” the system in a way reflecting the secretory pathway requirements of that cell type. The structure of the compartments present at steady state are a consequence of the way the system is “set”. An important aspect of setting the ground state of the system is the relative rate of anterograde flux with respect to retrograde recycling. The structure of the Golgi apparatus is highly variable between different cell types because different control points are dominant in these different systems. If we suppose that BFA perturbs one of these central regulators,

the equilibrium of the whole system will change upon treatment with BFA. The effects of BFA in different cell types, even though it affects the same regulator, will differ because the initial state of each system differs. Cells in culture are dividing and since retrograde transport is up-regulated during mitosis, this pathway may be naturally more developed in these cell types. It is interesting to note in this context that some cancer cell lines have a BFA-like phenotype in the sense that normally Golgi-resident glycosyl-transferases are mislocalized to the rough ER (Egea *et al.*, 1993). In contrast, the “raison d’être” of pancreatic acinar cells is secretion, so it is reasonable that Golgi-ER retrograde transport would be less developed compared to the anterograde flux. A similar bias towards anterograde transport is likely to be the case for *S. cerevisiae* cells in exponential growth. If we assume that BFA inhibits anterograde transport at the level of the ER in all of these systems, but does not inhibit retrograde transport, and we assume that the mechanism of anterograde transport is forward membrane flux, it is possible to explain the different phenotypes observed at the morphological level. In all cases, renewal of the Golgi apparatus from the ER is blocked, and depending on the strength and nature of retrograde flow in each situation, different amounts of Golgi structure will be left. In the rat spermatid, secretion is slow and there may be little or no retrograde transport. In this case, only accumulation of ER (behind the anterograde block point) occurs.

Studies of the effects of BFA on other cellular organelles are similar in several respects to those observed in the ER-Golgi system. The primary morphological effect of BFA in all systems is to cause the formation of tubular structures; the Golgi apparatus, TGN and lysosomes all extend tubular processes in the presence of BFA. Second, organelle identity is lost due to inappropriate fusion events, but not in an entirely non-specific fashion. The *cis*, medial and *trans* Golgi compartments fuse with the ER, but not with other organelles such as the TGN. The TGN fuses specifically with endosomes, and the lysosome remains as a distinct organelle. A final common theme in BFA effects at different levels within the cell is that trafficking out of the fused system can be blocked, whereas transport within a system continues. For the ER-Golgi system, retrograde transport continues, as well as some ER-ERGIC intermediate compartment transport, but transport out of the system is blocked. Transcytosis in MDCK cells treated with BFA is blocked, and transport from the cell surface to the lysosome is blocked in some cases. These common aspects of the morphological effects of BFA at different levels within the cell suggests that it is affecting a similar basic process in membrane dynamics and trafficking in different organellar systems. Another common feature of BFA treatment at all levels of the exocytic and endocytic pathways is the variability in effects of the drug

in different cell types. These observations imply that the targets of BFA in post-Golgi compartments are likely to be central regulators of membrane flow patterns, similar to those described above for the ER-Golgi system, that reflect the exocytic/endocytic requirements of a given cell type.

4. MOLECULAR EFFECTS OF BFA

4.1. BFA Causes the Rapid Release of the COPI Coat from Golgi Membranes

The first clue to the action of BFA at the molecular level was the demonstration that the COPI subunit β -COP (known at the time as the 110 kD protein) was released from Golgi membranes into the cytoplasm. This effect was found to be extremely rapid: after only 20 seconds redistribution had begun and was complete after 1 to 2 minutes (Donaldson *et al.*, 1990). Hence β -COP release from the Golgi was clearly one of the primary effects of BFA and the relationship of this event to the morphological effects of BFA described above was the subject of intense study in the early 1990's.

At this time, the biochemical requirements for transport through the Golgi apparatus were being defined using a cell-free *in vitro* assay developed in the laboratory of J. Rothman (Balch *et al.*, 1984; Fries and Rothman, 1980). This assay led to the purification of a complex, called COPI (membrane-associated form) (Serafini *et al.*, 1991b) or coatomer (for the cytosolic form, from coat protomer) (Waters *et al.*, 1991) that forms the proteinaceous coat of non-clathrin-coated (COPI) vesicles derived from Golgi membranes. COPI consists of 7 subunits (α , β , β' , γ , δ , ϵ , ζ) which under certain conditions is also associated with the low molecular weight GTP-binding protein ARF (for ADP ribosylation factor) (Serafini *et al.*, 1991a). In the laboratory of T. Kreis, a 110 kD microtubule-binding protein associated with the Golgi apparatus had been identified, and was found to be identical to β -COP (Duden *et al.*, 1991). Immunoelectron microscopy indicated that β -COP was localized to vesicular profiles and cisternae of the Golgi apparatus. BFA was shown to inhibit COPI binding to membranes in the *in vitro* Golgi transport assay, and to prevent formation of COPI-coated vesicles (Orci *et al.*, 1991). Instead, purified Golgi cisternae were transformed into anastomosing tubular networks in the presence of BFA. These tubules had the capacity to fuse, and in fact transport of VSV G protein monitored by this *in vitro* assay was essentially unaffected by the presence of BFA (Elazar *et al.*, 1994; Taylor *et al.*, 1994). The reason was clear: the tubules formed in the presence of BFA allowed intermixing of the contents

of the donor and acceptor cisternae through tubular connections between them. The conclusion from this work was that the formation of COPI vesicles was required *in vivo* for forward transport from the ER, and because BFA inhibited COPI binding to membranes, anterograde transport from the ER was consequently blocked. However, the fact that transport *in vitro* could occur just as efficiently without COPI vesicles as in their presence brought into question the nature of the role of COPI in anterograde ER-Golgi transport. The question is still not answered, despite an enormous amount of effort invested in examining the *in vitro* and *in vivo* roles of COPI. The current idea is that in fact the role of COPI is indirect (Gaynor *et al.*, 1998b). It is postulated that ARF is involved directly only in retrograde Golgi-ER transport, but since the anterograde transport machinery must be recycled to assure successive rounds of anterograde transport, defects in this recycling pathway could block anterograde transport *in vivo* (Gaynor *et al.*, 1998b).

There is controversy over the idea that COPI, once released from Golgi membranes, remains in a dispersed soluble form upon BFA treatment. Several studies showed that in pancreatic acinar cells, β -COP was not found dispersed in the cytoplasm but rather concentrated in novel structures associated with the ER. The significance of these observations is not clear, but may reflect part of the cycle of action of β -COP that is more highly developed in this cell type than in others (Hendricks *et al.*, 1993; Oprins *et al.*, 1993; Orci *et al.*, 1993).

4.2. BFA Inhibits Guanine Nucleotide Exchange on ARF

ARF (specifically mammalian ARF1) appeared on the BFA scene with the demonstration that it, like β -COP, was also released from Golgi membranes within minutes of BFA treatment (Donaldson *et al.*, 1991). At about the same time, it was shown that mammalian ARF1 co-purified with COPI when prepared from isolated Golgi membranes treated with GTP γ S (Serafini *et al.*, 1991b). Since it had been previously shown that binding of β -COP to membranes was affected by GTP γ S and aluminium fluoride (AlFn) (Donaldson *et al.*, 1991), these observations suggested that ARF1 might be regulating β -COP binding to Golgi membranes via its GTPase cycle and that BFA might affect this process. This prediction was elegantly verified by J. Donaldson and colleagues who demonstrated first that binding of mammalian ARF1 to membranes was a prerequisite to binding of β -COP, and that it was exclusively the former step that was inhibited by BFA (Donaldson *et al.*, 1992a). Secondly, along with Helms and Rothman, Donaldson *et al.* demonstrated that BFA inhibited guanine nucleotide exchange on ARF1 catalyzed by Golgi membranes (Donaldson *et al.*, 1992b; Helms and

Rothman, 1992). These results were supported by subsequent work (Palmer *et al.*, 1993; Randazzo *et al.*, 1993). Hence one molecular target of BFA is likely to be either an ARF guanine nucleotide exchange factor (GEF), or a protein associated with such a factor located on Golgi membranes. Confirming the idea that failure to carry out exchange on ARF1 is a major factor in the cellular effects of BFA, it was shown that expression of the dominant negative mutant ARF1^{T31N} in mammalian cells led to a phenotype identical to that of BFA treatment (Dascher and Balch, 1994).

4.3. BFA Causes the Release of Many Golgi-Associated Proteins from Membranes

Initially, it was assumed that the effects of BFA could be explained solely by its inhibition of COPI binding to membranes, but the identification of ARF exchange as the target of BFA renders this conclusion unlikely at best. Given that ARF is a regulatory protein with multiple effectors in addition to COPI, blocking activation of ARF will inhibit activation of all of these downstream effectors. Indeed, numerous studies have shown that β -COP release from Golgi membranes can be dissociated from the effects of BFA on Golgi disassembly (Ivessa *et al.*, 1995; Kok *et al.*, 1998; Mironov *et al.*, 1997; Scheel *et al.*, 1997). In addition, subsequent studies showed that BFA treatment causes release of a large number of different peripherally associated Golgi membrane proteins into the cytoplasm and not only COPI. These include a GTPase activating protein for ARF, ARF GAP, which was shown to be released from the Golgi (where it co-localized with β -COP) within minutes of BFA treatment (Cukierman *et al.*, 1995). Arfaptin1, another putative ARF effector, was also shown to be released rapidly into cytosol upon BFA treatment (Kano *et al.*, 1997). However, although Arfaptin1 was identified in a two-hybrid screen using a GTP-bound ARF mutant as bait, and it was shown to be an inhibitor of ARF function *in vitro*, its potential role as an effector of ARF has not been confirmed nor is its physiological role known (Tsai *et al.*, 1998).

Another regulatory protein affected by BFA treatment is Cdc42Hs, a member of the Rho family of small GTPases involved in the establishment and maintenance of cell polarity. After one minute of BFA treatment, Cdc42Hs was redistributed from its normal Golgi location in NR-6 fibroblast cells into the cytoplasm (Erickson *et al.*, 1996). Oxysterol binding protein PH domains bind to Golgi membranes, and are released within two minutes of BFA treatment (Levine and Munro, 1998). Oxysterol binding proteins bind to the Golgi apparatus in a regulated manner, upon binding of oxygenated derivatives of cholesterol, and their PH domains bind with high affinity and very specifically to PI(4,5)P2 both in Golgi membranes

and purified liposomes (Levine and Munro, 1998). The LDLC protein was identified as the product of a gene mutated in a cell line selected for defective low density lipoprotein (LDL) receptor activity (Podos *et al.*, 1994). The *ldlc* mutant cells have global defects in medial and *trans* Golgi glycosylation enzyme function. Although its precise function is not known, the LDLC protein was shown to be rapidly released from Golgi membranes upon BFA treatment, with the same kinetics as β -COP (Podos *et al.*, 1994). Another set of proteins released within minutes of BFA treatment include spectrin, ankyrin and p200/myosinII. Spectrin and ankyrin are proteins that interact with actin and are likely to be involved in maintaining the structure of the Golgi apparatus (Beck and Nelson, 1998). p200/myosinII, which also interacts with actin, has been shown to be part of a coat for vesicles released from the TGN (Musch *et al.*, 1997; Narula *et al.*, 1992; Narula and Stow, 1995). Since these proteins are released very rapidly from Golgi membranes, it is likely that their binding to the Golgi is regulated by the cycle of GDP/GTP binding to ARF, but this has not been directly demonstrated.

The adaptor complexes AP-1, AP-3 and more recently AP-4 (distinct coat complexes on TGN-derived vesicles) are all released from trans-Golgi/TGN membranes rapidly upon BFA treatment. As for COPI, the reason for this effect is that ARF1 regulates binding of the AP-1 and AP-3 coat complexes to Golgi membranes (Hirst and Robinson, 1998; Ooi *et al.*, 1998). This is likely to be the case for AP-4, but it has not yet been demonstrated directly (Dell'Angelica *et al.*, 1999). A close homologue (γ 2-adaptin) of the AP-1 complex component γ -adaptin has recently been identified. Remarkably, γ 2-adaptin, which is 70% identical to γ -adaptin, is not released from Golgi membranes by BFA treatment (Lewin *et al.*, 1998; Takatsu *et al.*, 1998). This result demonstrates the high level of specificity of BFA-induced release of proteins from Golgi membranes.

A second class of Golgi membrane-localized proteins are not released immediately from membranes upon BFA treatment, but only after the Golgi has fused with the ER and no longer exists as a separate organelle. These include the Rab6 small GTPase (Roa *et al.*, 1993) and the large coiled-coil proteins p230/golgin-245 (Kooy *et al.*, 1992) and GMAP-210/p210 (Infante *et al.*, 1999). The localization of other Rab proteins (Rab1A, Rab2, Rab4 and Rab9) are not affected by BFA (Roa *et al.*, 1993). The domain responsible for Golgi localization of p230/golgin-245 has recently been identified (the GRIP domain), and interestingly has been shown also to bind to Rab6 (Barr, 1999; Kjer-Nielsen *et al.*, 1999a; Kjer-Nielsen *et al.*, 1999b; Munro and Nichols, 1999). These results suggest that after fusion of the Golgi with the ER, there is a loss of Golgi identity distinct from the loss of peripheral membrane proteins from the Golgi that occurs immediately after BFA treatment.

5. THE Sec7 DOMAIN FAMILY OF ARF GUANINE NUCLEOTIDE EXCHANGE FACTORS

5.1. Identification of Sec7 Domain Proteins as ARF Exchange Factors

The "Sec7 domain" is an approximately 200 amino acid region of yeast Sec7p that was originally noted when other proteins with homology to this region were identified (Liu and Pohajdak, 1992; Shevell *et al.*, 1994; Zagulski *et al.*, 1995). As described above, SEC7 was identified by Schekman and colleagues in a selection for mutants defective in secretion in the yeast *S. cerevisiae*. The temperature sensitive sec7-1 and sec7-4 mutants identified in this selection accumulate abnormal Golgi structures when cells are shifted to the non-permissive temperature of 37°C (see above). This initial work and subsequent studies have established a requirement for Sec7p in ER-Golgi and intra-Golgi transport. Sec7p is localized to the Golgi apparatus, and is also present on the cytoplasmic surface of ER-Golgi transport vesicles (Franzusoff *et al.*, 1992; Franzusoff *et al.*, 1991). In fractionation experiments, Sec7p is present in both soluble and membrane-bound forms, suggesting that it cycles between membranes and cytosol (Franzusoff *et al.*, 1991). A requirement for Sec7p in the fusion of purified ER-Golgi transport vesicles with the Golgi acceptor compartment *in vitro* has been reported (Lupashin *et al.*, 1996), although in a different version of this assay using more highly purified components, Sec7p was shown to be dispensable (Barlowe, 1997).

Using a genetic selection designed to identify a guanine nucleotide exchange factor for ARF in *S. cerevisiae*, we identified the GE1 gene which encodes a 160 kD protein (Geal1p) with a centrally-located Sec7 domain (Peyroche *et al.*, 1996). Geal1p is 50% identical to a second yeast protein, Gea2p. Deletion of *GEA1* or *GEA2* in yeast leads to no detectable phenotype but the double deletion strain is inviable, indicating that Geal1/2p function is essential for vegetative growth of yeast cells. Like Sec7p, Geal1/2p is necessary for ER-through-Golgi transport in yeast, although Sec7p and Geal1/2p are not functionally interchangeable. Partially purified (His)6-tagged Geal1p stimulates GTP-GDP exchange on mammalian ARF1. Moreover, the Sec7 domain of Geal1p alone purified from *E. coli* is able to stimulate GTP/GDP exchange on myristoylated yeast ARF2 and mammalian ARF1 (Peyroche *et al.*, 1999). Hence, as was originally shown for the human ARNO protein (see below), the Sec7 domain of Geal1p is sufficient for ARF exchange activity.

Homologues of the yeast Geal1/2p proteins have been identified in *Homo sapiens* (GBF1, Mansour *et al.*, 1998), *Caenorhabditis elegans* (GenBank accession Z81475), and *Arabidopsis thaliana* (GNOM/Emb30p,

Busch *et al.*, 1996; Shevell *et al.*, 1994) and range in size from 160 to 210kD (see Figure 1). Mammalian GBF1 was identified as a protein that confers BFA resistance when over-expressed in mammalian cells (Yan *et al.*, 1994). GNOM/Emb30p was identified as the protein product of the gene mutated in the *gnom* pattern formation mutant of the *Arabidopsis* embryo (Jurgens 1995). Yeast Sec7p homologues (180–230kD) have been found in *Bos taurus* (p200) (Morinaga *et al.*, 1997), *Arabidopsis thaliana* (GenBank accession AL022604) and *Schizosaccharomyces pombe* (GenBank accession Z98602), although only p200 has been characterized functionally. These proteins range in size from 180–230kD, and have a significant level of homology throughout their lengths, especially in the centrally-located Sec7 domain (Morinaga *et al.*, 1997). The yeast and bovine Sec7 proteins are localized to the Golgi apparatus and function in ER-through-Golgi transport. The Gea/GBF/GNOM and Sec7/p200 proteins have little homology outside of a 300–400 amino acid region containing the Sec7 domain (Zagulski *et al.*, 1995).

The human protein ARNO was identified based on sequence similarity to the Gea1/2p proteins in a 200 amino acid region (Chardin *et al.*, 1996) and it was shown in this study that the Sec7 domain of ARNO purified from *E. coli* was sufficient to catalyze nucleotide exchange on ARF *in vitro*. Surprisingly, this activity was completely resistant to BFA *in vitro* (Chardin *et al.*, 1996). Cytohesin-1 was identified as a component of an integrin-mediated signalling pathway at the plasma membrane, and shown subsequently to have *in vitro* ARF exchange activity insensitive to BFA (Kolanus *et al.*, 1996; Meacci *et al.*, 1997). The mouse GRP1 protein, which has a structure very similar to that of both ARNO and cytohesin-1, was identified as a protein binding specifically to phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P₃] through its pleckstrin homology (PH) domain (Klarlund *et al.*, 1997). These three proteins are the founding members of a distinct class of ARF GEFs. In contrast to the large Gea and Sec7 ARF GEFs, the ARNO/cytohesin-1/GRP1 family members are much smaller (approximately 47 kD), and all contain an N-terminal coiled-coil, a central Sec7 domain and a C-terminal PH domain. The PH domain mediates membrane association by binding specifically to PtdIns(3,4,5)P₃ (or PtdIns(4,5)P₂) (Klarlund *et al.*, 1997; Nagel *et al.*, 1998; Paris *et al.*, 1997). ARNO3, the human homologue of GRP1, has been identified recently and shown to have characteristics similar to ARNO (Franco *et al.*, 1998). The crystal structure of the Sec7 domain of ARNO (Cherfils *et al.*, 1998; Mossessova *et al.*, 1998) and the NMR-derived solution structure of the Sec7 domain of cytohesin-1 (Betz *et al.*, 1998) have been determined, indicating that the Sec7 domain represents a structural as well as a functional unit. The structure of the Gea2p Sec7 domain in complex with nucleotide-free ARF has also been

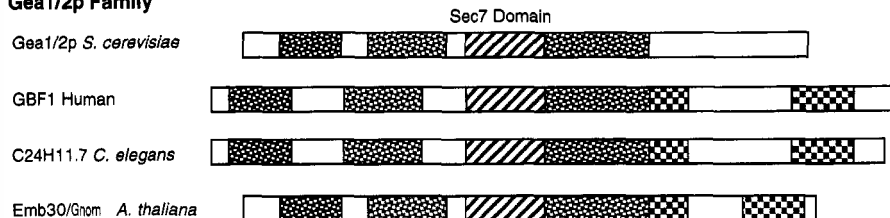
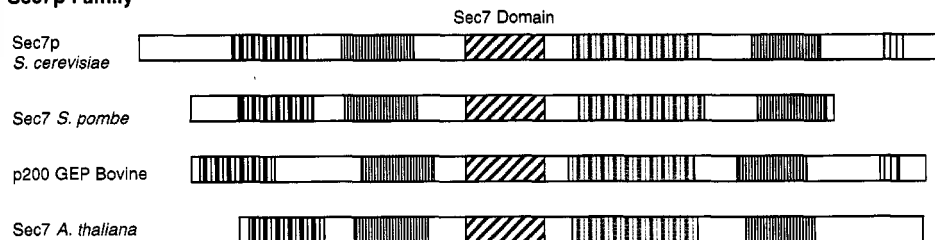
Gea1/2p Family**Sec7p Family****ARNO Family**

FIGURE 1. A schematic diagram showing the three families of Sec7 domain exchange factors that possess the motif “FRLPGE” in the core of the ARF binding site. The centrally-located Sec7 domain of each protein is indicated by a diagonally hatched box. The pleckstrin homology (PH) domain of the ARNO family is the only other sequence domain of defined function. The other shaded boxes indicate regions of sequence homology between members of each family, but their functions are not known. There is no highly significant region of homology (other than the Sec7 domain) between families. See text for references.

solved recently, and the similarity in structure of the ARNO and Gea2p Sec7 domains shows that different Sec7 domains adopt very similar conformations (Goldberg, 1998).

Two motifs within the structure of the Sec7 domain are intimately involved in ARF binding: the loop region between helices F and G, and helix H (Goldberg, 1998). The sequence of the F-G loop is invariant (consisting of the sequence FRLPGE) in all of the Gea/GBF/GNOM, Sec7/p200 and ARNO/cytohesin-1/GRP1 family members described above. A second class of Sec7 domain proteins (perhaps itself made up of several families) is more divergent in sequence, and in particular the sequence of the F-G loop does not correspond to FRLPGE. The best-characterized member of the latter class is the EFA-6 exchange factor that catalyzes exchange preferentially on mammalian ARF6 and affects the actin cytoskeleton at the plasma membrane (Franco *et al.*, 1999).

5.2. Different Sec7 Domain Proteins have Different Sensitivities to BFA

There is no ARNO homologue in *S. cerevisiae*, suggesting that ARNO function is specific to higher eukaryotes. Hence the "FRLPGE" class of Sec7 domain ARF exchange factors consists of the high molecular weight proteins conserved from yeast to humans, and the ARNO/cytohesin-1/GRP1 family of lower molecular weight factors unique to higher eukaryotic cells. To date, all members of the family of high molecular weight ARF exchange factors that have been tested have ARF exchange activity that is inhibited by BFA *in vitro*. These include partially purified full-length yeast Gea1p (ARF exchange activity half-maximally inhibited by 10 μ M BFA) (Peyroche *et al.*, 1996), bovine p200 purified from baculovirus (Morinaga *et al.*, 1996), and the Sec7 domain of yeast Sec7 purified from *E. coli* (Sata *et al.*, 1998). In contrast, all members of the ARNO/cytohesin/GRP family of small Sec7 domain GEFs have BFA-resistant ARF exchange activity (Chardin *et al.*, 1996; Meacci *et al.*, 1997).

Recent studies indicate that the differences in sensitivity to BFA are largely due to differences in sequence within the Sec7 domain itself (Peyroche *et al.*, 1999; Sata *et al.*, 1999). Peyroche *et al.* carried out random mutagenesis of the *GEA1* gene and selected mutants that grew better in the presence of BFA than the wild type. The mutations conferring BFA resistance fell into a 40-amino acid region of the Sec7 domain that overlaps the ARF binding site. Strikingly, this region contains multiple residues that differ between members of BFA-resistant and BFA-sensitive GEFs, but that are conserved among members within each family. Mutations in two of the residues within this 40 amino acid region of Gea1p (sensitive to BFA) or of ARNO (resistant to BFA) reversed the sensitivity of each to BFA

both *in vitro* and *in vivo* (Peyroche *et al.*, 1999). Sata *et al.* constructed chimeric Sec7 domains from the BFA-sensitive yeast Sec7p and the BFA-resistant cytohesin-1. They found that residues within the same 40 amino acid region identified by Peyroche *et al.* was responsible for conferring BFA resistance or sensitivity on the Sec7 domains they tested. In addition, two residues of this region (distinct from those identified by Peyroche *et al.*) were sufficient together to reverse the BFA sensitivity or resistance of the Sec7 domains of Sec7p and cytohesin-1 (Sata *et al.*, 1999). These studies demonstrate the high level of specificity of BFA for its target.

Having determined that it is the Sec7 domain itself that confers resistance to BFA, Peyroche *et al.*, constructed chimeric versions of the yeast Gea1p and Sec7p proteins in which the endogenous Sec7 domain of each protein was replaced by that of ARNO. These chimeric proteins, when expressed as the sole source of either Gea1p or Sec7p in yeast, conferred resistance to the effects of BFA. Strikingly, the double chimera strain grew almost as well in the presence of a high concentration of BFA as in its absence. Secretion into the medium as well as transport to the vacuole, blocked completely by BFA treatment of a wild type cell, was almost as efficient in the presence of BFA as in its absence in the double chimera strain. Hence the BFA inhibition of growth and secretion in yeast is largely due to inhibition of the Gea1/2p and Sec7p ARF exchange factors. Although it is not clear whether orthologues of Gea1/2p and Sec7 will turn out to be the sole targets of BFA in mammalian cells, given the high level of functional and sequence conservation among these GEFs, it is likely they will represent important BFA targets in higher eukaryotic systems as well.

5.3. Mechanism of Action of BFA Stabilization of an Abortive ARF-GDP-Sec7 Domain Protein Complex

The residues responsible for BFA sensitivity or resistance of Sec7 domains are found within the core of the ARF binding site, and these residues make direct contacts with residues of ARF (in some cases multiple contacts) (Goldberg, 1998; Peyroche *et al.*, 1996; Sata *et al.*, 1999). This observation led to the idea that BFA might be competing with ARF for binding to the Sec7 domain. Surprisingly however, Peyroche *et al.* found that BFA acts as an uncompetitive inhibitor, that is, its target is an ARF-Sec7 domain complex rather than the Sec7 domain itself (Figure 2). Furthermore, we found that the reaction intermediate recognized by BFA is an ARF-GDP-Sec7 domain protein complex, and that BFA acts to stabilize such a complex. Hence BFA acts by trapping ARF and its exchange factor in an abortive complex that blocks the reaction at an early stage before GDP release. In this way, BFA has the same effect as a dominant negative

mutation. Similar results have been obtained recently using the Sec7 domain of the mammalian Sec7p orthologue p200 (Sam Mansour and Paul Melançon, personal communication).

The idea of a small molecule acting to trap a reaction intermediate in an unproductive complex may have implications for other biological systems and may even have therapeutic applications. For example, it may be possible to screen for drugs that stabilize protein complexes in important signal transduction pathways (such as Rac and Ras) and hence block downstream events that lead to cancer (Chardin and McCormick, 1999). BFA is the only known inhibitor of an exchange reaction for small GTP-binding proteins, and so the demonstration that it blocks an early step in the exchange reaction may provide insight into the general mechanism of nucleotide exchange on small G proteins.

Knowing that BFA acts by stabilizing an ARF-GDP-Sec7 domain protein complex sheds light on some previously difficult-to-explain observations. The complex recognized and stabilized by BFA is one of the first intermediates on the nucleotide exchange reaction pathway and precedes the formation of the complex with nucleotide-free ARF (Figure 2). It has been observed that the concentrations of BFA used to inhibit nucleotide exchange on ARF *in vitro* vary over approximately two orders of magnitude. The uncompetitive inhibition mechanism of action of BFA could help to explain this fact. The ARF-GDP-Sec7 domain complex recognized by BFA is likely to be present in very limiting quantities *in vitro*, and therefore the half-maximal inhibitory concentration for a given reaction will be much higher than the true affinity of BFA for its target complex. Although the mechanism of action of BFA provides a major explanation for *in vitro* differences in sensitivity to the drug, it is possible that other factors may also be involved.

In certain mammalian systems, it has been noted that a higher level of BFA sensitivity is observed for the effects of the drug *in vivo* compared to its effects on *in vitro* reactions. For example, the BFA concentration required to inhibit COPI binding to Golgi membranes and induce tubule formation *in vitro* is approximately 10-fold higher than that required for similar effects *in vivo* (Orci *et al.*, 1991). The half-maximal inhibitory concentration for the ARF exchange activity of purified Golgi membranes from CHO cells was shown to be 10 μ M (Donaldson *et al.*, 1992b), whereas 0.2–0.4 μ M BFA is sufficient to half-maximally inhibit survival of CHO cells (Torii *et al.*, 1995). A possible explanation for these differences is that *in vivo*, the inhibitory effect of BFA would be favored because the reaction intermediate is present at higher levels than in the corresponding *in vitro* situation. This could be due to a higher local concentration of exchange factor and ARF-GDP at their site of action *in vivo*, or to stabilization of the target complex

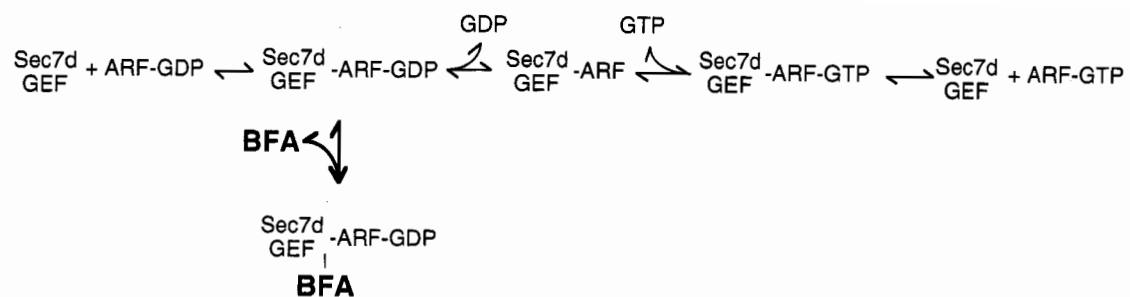


FIGURE 2. The mechanism of action of Brefeldin A (BFA). BFA acts as an uncompetitive inhibitor by binding to and stabilizing an ARF-GDP-Sec7 domain protein complex. This abortive complex prevents progression of the exchange reaction. GEF stands for guanine nucleotide exchange factor.

through the action of other proteins or membrane lipids involved in the exchange reaction *in vivo*. Hence knowledge of the mechanism of action of BFA will provide new avenues to identify potential partners or specific types of lipids that influence the ARF activation reaction *in vivo*.

6. CONCLUSION

BFA was the first agent that demonstrated the highly dynamic nature of the ER-Golgi system. The profound effects of BFA on intracellular membrane dynamics indicate that its targets are central regulators of this process. The identification of the Sec7 domain ARF exchange factors as major targets of the drug point to the importance of ARF in membrane dynamics/protein transport. How can we place ARF and its regulators into a model for ER-through-Golgi transport that takes into consideration the diverse *in vivo* effects of BFA? The morphological and biochemical analyses of BFA-treated cells discussed above indicate that BFA is blocking exit of proteins from the ER (or in a compartment closely associated with the ER). This transport block is accompanied by an inappropriate activation of Golgi glycosylation enzymes in the ER. These observations lead to the following speculative model for the role of ARF in the secretory pathway. I assume that regulated forward membrane flux is the driving force for anterograde transport through the secretory pathway (as proposed by Rambourg and Clermont), and that glycosylation enzymes are continually cycling between the Golgi and the ER or an ER-associated compartment. ARF acts to "hold up" the anterograde movement at the same time as it "holds up" and activates a specific set of glycosylation enzymes. Blocking membrane flow will result in accumulation of membrane behind the block (in the same way as a lake builds up behind a dam in a river) and hence in the physical appearance of a compartment. The dual action of holding up both forward membrane flow and a particular set of glycosylation enzymes will result in a compartment with a characteristic composition of modification enzymes. A problem raised by any type of "flow" model is how glycosylation enzymes can be concentrated in a specific location within what is imagined to be a continuous gradient of membrane. One potential solution is to suppose that the hold-up portions of a cycling glycosylation enzyme are long compared to its "round trip"; then at equilibrium, most of the enzyme will be present at one or a few "hold-up" points. In support of this idea is that transport *per se* can occur very rapidly. In yeast, proteins can transit the whole secretory pathway in just minutes (Gaynor *et al.*, 1998a). In mammalian cells, perturbations of the system such as BFA treatment reveals that cells have the capacity to transport all of the Golgi population of a given enzyme back to

the ER in just 10–15 minutes. If we assume that one regulator, such as ARF, co-ordinately holds up membrane flow and the progress of a specific set of glycosylation enzymes, the result will be compartments with characteristic markers. The degree to which each aspect of the block (to membranes flux vs. glycosylation enzyme cycling) is tight will determine how spread out or restricted the distribution of a given enzyme will be. This idea nicely explains why the distribution of the same enzyme differs in different cell types: the precise nature of the coregulation of membrane flow and glycosylation enzyme cycling will depend on the cell type (which itself would depend on the activity of that particular cell and its secretory needs). It would seem reasonable in the above model to keep the glycosylation enzymes inactive during their return cycle. I postulate that ARF controls this aspect as well by inhibiting the activity of glycosylation enzymes during their cycle and only activating them at a precise point. That ARF is in fact responsible for such a process is suggested by the fact that ARF inactivation leads to inappropriate activation of glycosylation enzymes (i.e. in the ER in BFA-treated cells). Yeast *gea* mutants (defective in the *Gea1p* and *Gea2p* ARF GEFs) as well as *arf* mutants have a similar phenotype (Anne Peyroche, Alain Rambourg and C.L.J., unpublished data). The improper regulation of glycosylation enzyme activation and inhibition could explain why these mutants have severe glycosylation defects. Another important feature to incorporate into this model is the co-ordination of membrane flux with anterograde cargo movement. This process is likely to involve cargo receptors that guide or escort proteins through the secretory pathway (Herrmann *et al.*, 1999). At the level of the ER, the COPII coat is clearly essential for this process, in conjunction with the ARF-like GTPase *Sar1p*. Whether or not ARF is involved in this process as well is an open question. ARF has a clear role in the function of the COPI coat, which is important for sorting of glycosylation enzymes and transport machinery away from anterograde cargo in post-ER compartments (Gaynor *et al.*, 1998b). It is possible that ARF co-regulates glycosylation enzyme activity with anterograde cargo movement through interaction or regulation of cargo receptors. This regulation might be achieved either through direct interaction with cargo receptors or through ARF-COP1 interactions. The latter possibility is supported by the fact that COPI has been shown to have cargo-specific effects on ER-Golgi transport, and, like *arf* and *gea* mutants, *copl* mutants have defects in glycosylation of cargo proteins.

A fundamental issue in the regulated forward membrane flux model is defining in molecular terms the force generating forward membrane flow. The endoplasmic reticulum is the site of synthesis of the bulk of intracellular lipids, and it has been estimated that it takes only four minutes for half of the lipid content of the ER to be transported to the Golgi in mammalian

cells (Karrenbauer *et al.*, 1990). It is likely that continued synthesis of lipids, and their regulated channelling into ER exit sites for anterograde transport, coupled with anterograde protein cargo concentration at these sites are key events. J. Lippincott-Schwartz and colleagues have proposed the idea that there is a higher level of energy in the Golgi membrane system compared to the ER membrane system, and that the increased energy level is produced by cycles of nucleotide hydrolysis on GTP-binding proteins such as ARF (Sciaky *et al.*, 1997). One important role of ARF is to recruit COPI to membranes which acts to sort anterograde cargo from resident and recycling ER and Golgi proteins. Hence the energy dispensed by ARF is used to produce a more highly ordered system. Another important factor that could contribute to forward anterograde membrane flux is the regulation of the rate and nature of lipid synthesis, and the consequent setting in motion of regulatory cascades at the membrane level (that co-ordinate membrane transformation events with protein cargo progression). An attractive idea, put forward by Emr and colleagues for the Golgi-vacuole pathway, is that anterograde cargo concentration activates a key lipid-modification enzyme (the PI(3) kinase Vps34p in the case of Golgi-vacuole transport), which results in the localization of a number of different proteins and regulators to the locally modified membrane region (Burd and Emr, 1998). Applying this model to the ER-Golgi system, we would postulate that concentration of anterograde-bound cargo in the ER would activate a series of membrane-modification events that would lead to anterograde movement of protein cargo. The importance of lipid second messenger cascades as regulatory components of constitutive secretion has been reviewed previously (De Camilli *et al.*, 1996).

How can we visualise the coupling of forward membrane flux to cargo protein transport? Rambourg and colleagues noted in their studies of secretory cells that concentration of secretory material at the *trans* Golgi/trans tubular network was always tightly coupled to membrane transformation events (saccules—fenestrated saccules—tubules). Their studies suggest that segregation (concentration) of secretory material is in fact accomplished by its “draining” from tubular elements that become progressively thinner as the secretory material becomes more concentrated (Rambourg and Clermont, 1990). More recently, Rambourg and colleagues have deduced a similar mechanism occurring at the level of the ER in yeast (Morin-Ganet *et al.*, 1998). Hence a general theme that emerges is the intimate coupling of cargo concentration to membrane fenestration and tubulation. The molecules that mediate and regulate this progressive fenestration and tubulation of membranes and the factors that couple these membrane transformation events to concentration of cargo, will be key players involved in the mechanisms generating forward membrane flux. The effects

of BFA in different systems demonstrate that its targets are central regulators of these events. The recent data showing that BFA stabilizes an abortive Am-GDP-Sec7-domain ARF GEF complex indicates that ARF and its regulators are indeed key players in the regulation of intracellular membrane dynamics. The exact role that ARF and the ARF GEFs play in coupling membrane transformation events to concentration of anterograde-bound cargo and its correct modification by glycosylation enzymes along the exocytic pathway will await further studies.

ACKNOWLEDGMENTS. I am especially grateful to Alain Rambourg for opening my eyes to the world of organelles inside the eukaryotic cell. I am grateful to Frangois Képès for introducing Alain Rambourg to the yeast *Saccharomyces cerevisiae* and for introducing me to Alain.

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Chapter 7

Membrane Fusion Events during Nuclear Envelope Assembly

Philippe Collas* and Dominic Poccia

INTRODUCTION: THE NUCLEAR ENVELOPE IS A DYNAMIC STRUCTURE

The controlled environment of the cell nucleus is separated from the cytoplasm by the nuclear envelope (NE). The NE consists of a double membrane fenestrated by nuclear pores and underlaid by the nuclear lamina (Dingwall and Laskey, 1992; Marshall and Wilson, 1997b). The lamina is built into a network of type V intermediate filament proteins called A/C- and B-type lamins (Aebi *et al.*, 1986; Fisher *et al.*, 1986; McKeon *et al.*, 1986). The NE is believed to be essential for a variety of nuclear functions, such as macromolecule transport in and out of the nucleus, chromatin organization and DNA synthesis (Laskey *et al.*, 1996).

Nuclear membranes are organized into three distinct domains, each associated with specific integral and peripheral polypeptides. (1) The outer nuclear membrane is continuous with the rough endoplasmic reticulum (ER) and functions as a part of the ER. It is studded with ribosomes and

PHILIPPE COLLAS Institute of Medical Biochemistry, University of Oslo, PO Box 1112 Blindern, 0317 Oslo, Norway. **DOMINIC POCCIA** Department of Biology, Amherst College, Amherst, MA 01002, USA. *Author for correspondence.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

contains proteins involved in rough ER function (Rapoport *et al.*, 1996). (2) The inner nuclear membrane is associated on its nucleoplasmic side with the lamina. Since the lamina is discontinuous, the inner nuclear membrane also directly interacts with chromatin, and thus harbors a set of lamin- and chromatin-binding integral proteins. All inner membrane integral proteins characterized to date have nucleoplasmic domains of less than 50 kDa and one or more transmembrane domains. Lamina-associated polypeptides (LAPs) 1 and 2 bind lamins and chromosomes (Foisner and Gerace, 1993; Furukawa *et al.*, 1998; Furukawa *et al.*, 1995; Gerace and Foisner, 1994; Martin *et al.*, 1995). A human protein called thymopoietin b has been shown by cDNA cloning to be essentially identical to LAP2 (Harris *et al.*, 1994). The lamin B receptor (also called LBR/p58/p54) (Bailer *et al.*, 1991; Schuler *et al.*, 1994; Simos and Georgatos, 1992; Worman *et al.*, 1990; Worman *et al.*, 1988; Ye and Worman, 1994) has been cloned in chicken and human. A putative homologue of LBR (p56) has also been characterized in echinoderms (Collas *et al.*, 1996). The N-terminal domain of LBR binds lamin B *in vitro* as well as chromatin protein HP1 (Worman *et al.*, 1988; Ye and Worman, 1994; Ye and Worman, 1996), and is believed to play a major role in the attachment of nuclear vesicles to chromatin. Otefin is a strongly anchored, but peripheral, protein of the inner nuclear membrane of *Drosophila* nuclei (Ashery-Padant *et al.*, 1997). (3) The pore membrane domain connects the outer and inner nuclear membranes and carries integral membrane proteins of the nuclear pore complex (NPC) such as gp210 (Gerace *et al.*, 1982) and POM121 (Hallberg *et al.*, 1993). Additional proteins of the NE have been identified, such as emerin and the so-called MAN antigens. Emerin has been localized in nuclear membranes of human cells, although whether it is restricted to the inner or outer membrane is not known (Nagano *et al.*, 1996). MAN antigens are non-lamin constituents of the nuclear lamina of vertebrate cells, that have been proposed to function in the maintenance of nuclear architecture (Paulin-Levasseur *et al.*, 1996).

The nucleus is a highly dynamic structure that periodically disassembles and reassembles at mitosis (Cox and Hutchison, 1994; Gerace and Foisner, 1994; Marshall and Wilson, 1997b; Wiese and Wilson, 1993). Disassembly and reassembly of the NE has been investigated in cultured mammalian cells by transmission electron microscopy (TEM) (Zatsepina *et al.*, 1977; Zatsepina *et al.*, 1982) and fluorescence microscopy (Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Meier and Georgatos, 1994). Mitotic NE breakdown entails the dispersion of nuclear membranes into the cytoplasm, solubilization of A/C type lamins, release of B-type lamins mostly in a membrane-bound form, and dismantling of nuclear pores. NE disassembly is associated with mitotic-specific phosphorylation of nuclear membrane proteins (Courvalin *et al.*, 1992), nuclear pores (Favreau *et al.*,

1996) and of the lamina (reviewed in Fields and Thompson, 1995) (see also Pfaller and Newport, 1995; Pfaller et al., 1991). During anaphase, populations of cytoplasmic membrane vesicles ("nuclear vesicles") bind to the chromatin, and subsequent fusion of chromatin-bound vesicles seals the new nuclear membranes. Further growth of the nucleus (and of the NE) proceeds during interphase as the nucleus acquires the ability to support DNA replication and transcription.

The dynamic nature of the NE is also illustrated by the transformation of the sperm nucleus into a male pronucleus at fertilization (see reviews by Poccia and Collas, 1996; Poccia and Collas, 1997; Poccia and Green, 1992). Morphological processes of male pronuclear assembly inside the egg cytoplasm are to some extent similar to those of somatic nuclear reconstitution after mitosis. They include removal of the nuclear membranes, dispersal of the sperm nuclear lamina, decondensation of the chromatin, reassembly of a new NE including pores and a lamina, and expansion of the newly formed pronucleus (see Figure 1).

Whereas components mediating membrane vesicle binding to chromatin are being identified, the molecules mediating nuclear vesicle fusion remain largely unknown. In contrast, molecular players involved in docking and fusion of cytoplasmic vesicles in endocytic and exocytic trafficking pathways are being rapidly unraveled. Here, we review and discuss our current knowledge on nuclear vesicle fusion. Several issues are addressed, including: (1) the multistep assembly of the NE; (2) the biochemical requirements for fusion of nuclear vesicles; (3) evidence for the involvement of small GTPases in nuclear vesicle fusion; (4) analogies between nuclear vesicle fusion and fusion events in intracellular membrane trafficking; (5) the controversial role of Ca^{2+} in NE assembly; and (6) evidence that some nuclear vesicles may harbor specific fusigenic elements.

1. ASSEMBLY OF THE NUCLEAR ENVELOPE IS A MULTI-STEP PROCESS

1.1. Nuclear Reconstitution in Cell-Free Systems as Tools to Study Nuclear Envelope Assembly

Much of our knowledge on the morphological and biochemical transformations driving to nuclear assembly has benefited from the development of cell-free nuclear reconstitution systems. Such systems consist primarily of a (i) cytosolic (membrane-free) or cytoplasmic (membrane-containing) extract prepared from eggs or somatic cells, (ii) a source of DNA or chromatin, such as plasmid DNA (Newport, 1987), mitotic chromosomes (Burke

and Gerace, 1986; Nakagawa *et al.*, 1989) or most commonly, permeabilized sperm nuclei (Cameron and Poccia, 1994; Lohka and Masui, 1983; Longo *et al.*, 1994; Ulitzur and Gruenbaum, 1989), (iii) cytoplasmic membrane vesicles, and (iv) an energy-generating system. Preparations from activated *Xenopus* eggs (Lohka and Masui, 1983; Wilson and Newport, 1988), *Drosophila* embryos (Berrios and Ailion, 1990; Ulitzur and Gruenbaum, 1989), activated oocytes of surf clam (Longo *et al.*, 1994) and sea urchin (Cameron and Poccia, 1994; Collas and Poccia, 1995c), as well as from mitotic mammalian (Burke and Gerace, 1986) and avian (Nakagawa *et al.*, 1989) cells, have been shown to support nuclear reconstitution.

The formation of a functional NE is a step-wise process involving targeting and binding of nuclear vesicles to the chromatin surface, fusion of these vesicles to seal the envelope, and a newly identified process of flattening of the vesicles and “smoothing” of the nuclear membrane (Wiese *et al.*, 1997). Concomitantly, NPCs assemble, an event requiring fusion of outer and inner nuclear membranes. Subsequent nuclear growth is accompanied by expansion of the NE, which occurs by fusion of additional vesicles and polymerization of the newly assembled lamina (Collas *et al.*, 1995a; Collas and Poccia, 1995b). Concomitantly, the establishment of nuclear structures such as nuclear matrix, DNA replication centers, and nuclear bodies contributes to rendering the nucleus functional for replication and transcription (Marshall and Wilson, 1997b). A summary of the sequences of NE assembly around sea urchin sperm chromatin in a fertilized egg extract is shown in Figure 1. Several reviews on nuclear assembly depict diagrams of

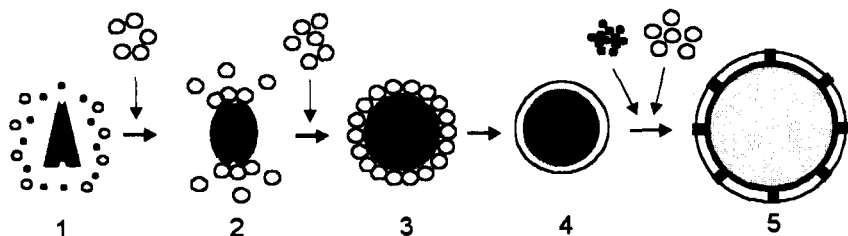


FIGURE 1. The different stages of sea urchin male pronuclear envelope assembly in fertilized egg extract. (1) The fertilizing condensed sperm nucleus is stripped off its lamina and most of its nuclear membranes. (2) Binding of distinct sets of nuclear vesicles initiates near polar regions of the sperm nucleus called lipophilic structures (shown as cups on the decondensing chromatin; see section 1.3). (3) This initial vesicle binding event is followed by binding of the bulk of nuclear vesicles to the entire chromatin surface. (4) Fusion of chromatin-bound vesicles seals the nuclear membranes. (5) Swelling of the pronucleus requires fusion of additional membrane vesicles (circles), functional nuclear pores (squares), and import of soluble lamins (dots) to assemble a nuclear lamina (thick line).

somatic nuclear reconstitution (Gerace and Foisner, 1994; Lourim and Krohne, 1994; Marshall and Wilson, 1997b; Wiese and Wilson, 1993).

1.2. Targeting and Binding of Nuclear Vesicles to Chromatin

The first step of NE reassembly after mitosis or at fertilization is the targeting and binding of nuclear vesicles to chromatin. Whether these events require energy or cytosolic components differs between species. In *Xenopus*, vesicle binding occurs at 4°C, and is not dependent on cytosolic factors or nucleotides such as ATP or GTP (Newport and Dunphy, 1992; Wilson and Newport, 1988), indicating that energy is not required for the binding process. Absence of a cytosolic requirement for binding indicates that this process involves exclusively membrane and/or chromatin associated components. In the sea urchin, nuclear vesicle binding to chromatin requires both cytosol and ATP (but not ATP hydrolysis) (Collas and Poccia, 1995c), indicating that in this system vesicle targeting and binding involve as yet unidentified soluble factors. In both species, the binding ability of the majority of nuclear vesicles to chromatin is abolished by protease treatment of the vesicles, but resists high-salt extraction of vesicles (Collas and Poccia, 1996a; Collas and Poccia, 1996b; Wilson and Newport, 1988), suggesting that attachment of the bulk of nuclear vesicles to chromatin is mediated by proteins tightly anchored to the membrane.

The nature of the molecules mediating nuclear vesicle targeting and binding to chromatin, i.e., chromatin- and/or membrane-associated lamins, or integral membrane proteins, has been a source of controversy over the past twelve years. Recent evidence strongly argues, however, for an essential role of integral membrane proteins in anchoring vesicles to the chromatin surface. Two models of NE formation have emerged from experiments in cell-free nuclear reconstitution systems. In the first model, illustrated by *Drosophila* embryo and mammalian cell extracts, vesicle binding first requires initial attachment of peripheral membrane proteins such as lamins (Burke, 1990; Burke and Gerace, 1986; Glass and Gerace, 1990; Lourim and Krohne, 1993; Ulitzur *et al.*, 1992) or nuclear pore components (Sheehan *et al.*, 1988). The second model, supported by cell-free studies in *Xenopus* (Meier *et al.*, 1991; Newport *et al.*, 1990) and sea urchin (Collas *et al.*, 1995a; Collas and Poccia, 1995b) involves lamin-independent targeting and attachment of nuclear vesicles to chromatin. Two studies have demonstrated that the integral membrane protein LBR, or its echinoderm analogue p56, is required for targeting and anchoring the bulk of nuclear vesicles to chromatin (Collas *et al.*, 1996; Pyrpasopoulou *et al.*, 1996). It is noteworthy that the latter studies provided the functional demonstration of earlier immunofluorescence observations of nuclear reassembly in

mitotic HeLa cells, where LBR-containing vesicles were shown to be targeted to chromosomes before lamin B (Chaudhary and Courvalin, 1993). The most recent data clearly support, therefore, a model of lamin-independent NE assembly mediated by direct attachment of integral membrane proteins to chromatin rather than through a lamin-dependent process.

1.3. Role of Lipophilic Structures (LSs) in Membrane Vesicle Binding to Chromatin

In the sea urchin, initial binding of vesicles to sperm chromatin in cytoplasmic extracts occurs at the two tips of the sperm nucleus, in the acrosomal and centriolar fossa regions (Figure 2). Sea urchin sperm nuclei permeabilized with 0.1% of the non-ionic detergent Triton X-100 lose their NE except for two specialized membrane remnants at the tip and the base of the nucleus, which could be labeled with a lipophilic dye. We therefore named these structures "lipophilic structures" or LSs (Collas and Poccia, 1999). LSs correspond to portions of the sperm NE retained after fertilization *in vivo* (Longo and Anderson, 1968). It is at those tips that nuclear vesicle binding initiates, before progressing towards the equator of the nucleus and covering the entire chromatin surface (Figure 2). Upon fusion of chromatin-bound vesicles, the vesicles also fuse with the LSs which become incorporated into the nuclear membrane (Collas and Poccia, 1995c; Collas and Poccia, 1996a; Collas and Poccia, 1996b) (see below). Nuclear vesicle-LS interactions are mediated by peripheral (salt-extractable) membrane proteins of two specific sets of vesicles (Collas and Poccia, 1996a).

LSs are not mere membrane remnants that serve as targets for nuclear vesicle binding, but are structures required for NE assembly at least *in vitro* (Collas and Poccia, 1999; Collas and Poccia, 1996a). Extraction of

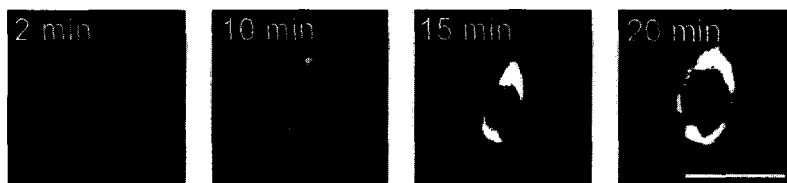


FIGURE 2. Polarized nuclear vesicle binding around decondensing sea urchin sperm chromatin in sea urchin egg extract. Nuclear vesicles of cytoplasmic extracts were labeled with the fluorescent lipophilic dye DiOC₆. Vesicles bind initially at the two poles of the conical nucleus (region of the LSs) and then progressively around the periphery. Time of incubation is indicated. Taken from Collas and Poccia (1995a) with permission.

demembranated nuclei with 1% Triton X-100 removes the LSs. The solubility characteristics of the solubilized material are not typical of normal membranes, and may originate from unusual lipid composition or association with proteins. Removal of LSs abolishes membrane binding to chromatin. When LSs are added back to stripped nuclei, they re-bind specifically to their sites of origin, at one or both nuclear poles. Unipolar LS reconstitution allows vesicle binding to one pole only, and bipolar reconstitution allows vesicle binding to both poles and formation of a complete NE (Collas and Poccia, 1999%). LSs are not just adhesive sites for promiscuous binding of vesicles, since not all egg vesicle fractions bind LSs or chromatin, nor do membranes extracted from sperm heads (Collas and Poccia, 1996a). LSs have been identified in all genera examined, including echinoderms, fish, amphibians and several mammals (Collas and Poccia, 1996a), arguing that they may be of general occurrence. A fundamental question that remains to be addressed is whether individual chromosomes also contain similar nuclear vesicle-targeting structures. Such structures could account for the assembly of nuclear membranes around individual chromosomes (forming so-called karyomeres) upon mitotic NE reassembly in rapidly dividing embryos (Lemaitre *et al.*, 1998).

1.4. Distinct Membrane Vesicle Populations Contribute to the NE

There is increasing evidence that NE assembly requires contributions from various cytoplasmic vesicle fractions, which may exhibit different biochemical and chromatin binding (as well as fusion) properties. Previous reports using biochemical fractionation (Lourim and Krohne, 1993; Vigers and Lohka, 1991; Vigers and Lohka, 1992) and immunofluorescence analyses (Chaudhary and Courvalin, 1993; Collas and Poccia, 1996b; Foisner and Gerace, 1993; Meier and Georgatos, 1994) argued that several distinct types of nuclear vesicles might contribute to the NE *in vitro*.

In the sea urchin, in addition to the sperm LSs, three populations of egg membrane vesicles (MV1, MV2 α and MV2 β) have been separated by buoyant density, that contribute to the male pronuclear envelope (Collas and Poccia, 1996b). Binding of each of these fractions to chromatin requires LSs. The fractions display distinct biochemical, binding and fusion properties, as summarized in Table 1. MV1 binds exclusively in the LS regions and is required for fusion of the vesicles to each other and to the LSs. MV2 β , the most abundant fraction in the egg cytoplasm, binds over the entire chromatin surface. MV2 β is enriched in the ER marker α -D-glucosidase and contains marker proteins such as lamin B receptor (LBR) and lamin B. MV2 α binds only in the LS regions, is enriched in the Golgi marker α -D-mannosidase and is required together with the MV1 fraction for fusion of

Table 1
Properties of sea urchin egg membrane vesicle fractions

Membrane fraction	Surface binding	Protease-sensitive	NEM-sensitive	Binding protein ^b	Fusigenic	Marker proteins
MV1	Tips ^a	Yes	Yes	PMP	Yes ^c	None
MV2 α	Tips ^a	Yes	No	PMP	No	Golgi ^d
MV2 β	All	Yes	No	IMP	No	ER ^e , LBR, Lamin B

^a Binding in the polar LS regions. ^b PMP, peripheral membrane protein; IMP, integral membrane protein.

^c Requires LSs to fuse. ^d α -D-mannosidase. ^e α -D-glucosidase.

MV2 β vesicles around the chromatin periphery. Together with the LSs, all three fractions are required to form a complete NE excluding high molecular weight dextrans (Collas and Poccia, 1996b).

NE assembly in *Xenopus* egg extracts also requires at least two nuclear vesicle populations, named NEP-A and NEP-B (Vigers and Lohka, 1991). Vesicles of NEP-B contain an ER marker and directly bind to chromatin. In contrast, those of NEP-A do not carry the ER marker and do not bind chromatin, but associate only with chromatin-bound NEP-B vesicles. NEP-A vesicles can fuse with one another and with NEP-B vesicles to form a nuclear membrane (Vigers and Lohka, 1991). Activities of NEP-A and NEP-B fractions are sensitive to proteases, sodium carbonate and detergents, but are distinguished by their sensitivity to high salt and to the alkylating agent *N*-ethylmaleimide (Vigers and Lohka, 1991). Involvement of distinct vesicle populations for *Xenopus* NE formation has recently been corroborated in detailed EM observations. Two morphologically distinct classes of vesicles were shown to contribute to the NE: vesicles bearing ribosomes, many of which bind directly to chromatin, and smooth vesicles that primarily associate with chromatin-bound membranes (Wiese *et al.*, 1997). The relationship of the latter classes of vesicles with those of NEP-A and NEP-B remains to be established (see discussion in Wiese *et al.*, 1997).

Recent preliminary data obtained in a cell-free somatic nuclear reconstitution assay support these earlier findings (PC; unpublished observations). Cytoplasmic vesicles purified from mitotic KE37 cell extracts (in collaboration with Dr. J.-C. Courvalin, CNRS, Institut Jacques Monod, Paris, France) and floated to density equilibrium in a sucrose gradient, produced three membrane fractions (MF-A, MF-B and MF-C; Figure 3A). MF-A, MF-B, MF-C and the residual pellet fraction represented 18%, 62%, 12% and 8% of input mitotic vesicles, respectively. The fractions displayed different protein patterns in an SDS-polyacrylamide gel (Figure 3B) and

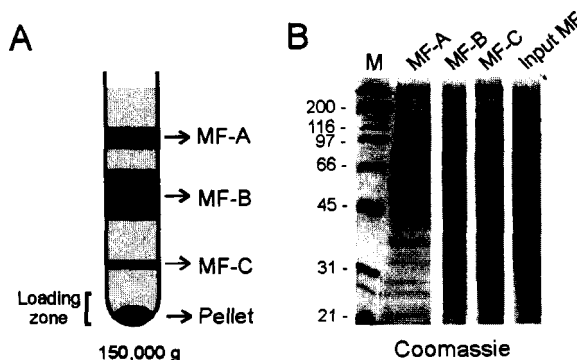


FIGURE 3. Fractionation of cytoplasmic membrane vesicles isolated from KE37 mitotic cell lysates. (A) Membrane fractions (MF) produced by floatation of total mitotic vesicles to density equilibrium by ultracentrifugation into a 2M (bottom)—0.2 M (top) sucrose step gradient. (B) Proteins of each fraction were resolved by 15% SDS-PAGE and stained with Coomassie blue. M, molecular weight standards, indicated in kDa.

distinct chromatin binding properties. In a cytosolic extract of interphase KE37 cells, MF-A and MF-B were found capable of binding to exogenously added demembranated HeLa cell nuclei, while MF-C was not (data not shown). Altogether, these observations argue for the contribution of several populations of nuclear vesicles to the NE, with distinct binding and perhaps fusion characteristics. They also suggest the existence of cytoplasmic membrane domains enriched in specific components—regardless of whether these membranes exist as a continuous network or as vesicle during mitosis—which may be important for NE assembly (Buendia and Courvalin, 1997; Chaudhary and Courvalin, 1993; Collas *et al.*, 1996; Collas and Poccia, 1996b; Wiese *et al.*, 1997).

2. FUSION OF NUCLEAR VESICLES

2.1. Sealing and Growth of the Nuclear Envelope

2.1.1. Fusion of the Bulk of Nuclear Vesicles

The formation of continuous nuclear membranes enclosing the chromatin requires fusion of chromatin-bound vesicles with one another. Additional fusion events take place after nuclear membranes have sealed, in order to incorporate vesicles necessary for nuclear membrane growth (Collas *et al.*, 1996; Collas and Poccia, 1995b; Wiese *et al.*, 1997; Wilson and

Newport, 1988) and, possibly, for the assembly of functional NPCs (Collas, 1999). Growth of the NE is associated with flattening of the fused nuclear vesicles onto the chromatin surface and “smoothing” of the NE (Wiese *et al.*, 1997). NE growth is also accompanied by the assembly of a new nuclear lamina (Collas *et al.*, 1995a; Collas and Poccia, 1995b), and follows the establishment of functional NPCs (Collas *et al.*, 1996). NE growth is associated with swelling of the nucleus to several times its diameter, and usually completes nuclear (or pronuclear) assembly (Collas *et al.*, 1995a; Collas and Poccia, 1995b; Wilson and Newport, 1988). Processes susceptible of regulating nuclear swelling have been discussed previously (Collas *et al.*, 1995a; Collas and Poccia, 1995b; Poccia and Collas, 1997) but remain speculative. It has been proposed that polymerization of the lamina drives expansion of the nucleus (Collas *et al.*, 1995a; Collas and Poccia, 1995b), while maximum nuclear volume is probably dictated, at least to some extent, by the amount of nuclear vesicles susceptible of being incorporated by fusion into the growing envelope (Collas and Poccia, 1995b; Wilson and Newport, 1988).

2.1.2. LS-Vesicle Fusion

In addition to fusing with one another, some nuclear vesicles also fuse specifically with LSs, which thereby become incorporated into the pronuclear envelope (Collas and Poccia, 199c; Collas and Poccia, 1996a; Collas and Poccia, 1996b). The purification of distinct cytoplasmic vesicle populations from sea urchin egg extracts has led to the identification of a unique fraction with fusigenic properties (Collas and Poccia, 1996b). Most fractions (MV2 α and MV2 β) exhibiting chromatin (MV2 β) or LS (MV2 α and MV2 β) binding properties are by themselves not capable of fusing with LSs, with one another, or with themselves. Vesicles of one fraction (MV1), however, are capable of fusing with LSs, and with one another but only in the presence of the LS. Curiously, MV1 does not fuse with MV2b, which makes up the bulk of nuclear membranes. LS-MV1-MV2 β fusion requires an “intermediate” brought by MV2 α , even though MV2 α by itself does not fuse with LSs or MV2 β . All fusion events require soluble factors from egg cytosol and GTP hydrolysis (Collas and Poccia, 1996b). It is clear from these observations that LSs alone cannot provide a general fusion signal propagated to any bound vesicles. In the absence of MV1, no fusion is detected, indicating that a critical component of the fusion signal or part of the fusion machinery resides in MV1 vesicles. The nature of the putative fusion signal and of the fusigenic components of MV1 are under current investigation in our laboratories.

2.2. A Retrograde Vesicular Transport Mechanism Implicated in Nuclear Vesicle Targeting to Chromatin and Fusion?

The contribution of Golgi-derived vesicles to the male pronuclear envelope is intriguing. Despite the absence of microtubule organizing center in the acrosomal fossa region of sea urchin sperm, it may still be possible that the participation of MV2 α together with MV1 represents an incipient Golgi assembly (Acharya *et al.*, 1995a; Acharya and Malhotra, 1995b; Love *et al.*, 1998). Both MV1 (non-ER-derived) and MV2 α (Golgi-derived) bind in the LS region and fuse with each other. Nevertheless, since MV1 does not carry any Golgi marker, another alternative is that fusion of MV2 α with MV1 and MV2 β (ER-derived) represents a tentative reconstitution of a retrograde fusion pathway (Lippincott-Schwartz *et al.*, 1990). Retrograde fusion is defined as the fusion of Golgi-derived vesicles back to the ER (Lord and Roberts, 1998). Golgi vesicles (in MV2 α) and vesicles of unidentified, but non-ER, origin (in MV1) may be necessary to supply fusogenic components for fusion of ER vesicles bound to chromatin. The inability of MV2 β vesicles to fuse on their own suggests that *in vitro* sea urchin nuclear assembly conditions (Collas and Poccia, 1996b) support retrograde fusion rather than “organelle-assembly” fusion.

2.3. Assays for Nuclear Vesicle Fusion

Nuclear vesicle fusion may be monitored directly by double direct fluorescence labeling of vesicles, phase contrast microscopy, TEM, and at a higher resolution level by field emission in-lens scanning EM (FEISEM). Fusion has also been monitored indirectly by exclusion of high molecular weight dextran from nuclei.

2.3.1. Fluorescence Evidence of Fusion

Since binding of numerous fluorescently labeled vesicles to the chromatin surface can result in a fluorescent rim around the nucleus (Collas and Poccia, 1995c), the use of a single fluorescent dye is not sufficient to assess vesicle fusion. As a result, an assay based the mixing of fluorescent lipophilic dyes was developed in the sea urchin to monitor nuclear vesicle fusion (Collas and Poccia, 1995c; Collas and Poccia, 1998). Two sets of vesicles pre-labeled green (with DiOC₆) or red (with DiI C₁₈) are bound to chromatin. Fusion of the green- and red-labeled vesicles is triggered by addition of GTP and monitored by mixing of the green and red dyes within the fused membranes (Collas and Poccia, 1996c). Before fusion, the fluorescence

patterns of green- and red-labeled vesicles are distinct. However, upon addition of GTP, fluorescence patterns in the green and red channels appear identical due to the redistribution of the labels within the fused membranes. Emission in the green channel also appears yellow-orange instead of green, an effect due to both green and red emission detected in the green channel. This observation was suggested to result from fluorescence energy transfer from DiOC₆ to DiIC₁₈, but this interpretation remains to be demonstrated. Regardless, orange fluorescence resulting from fusion depends on the close proximity of the two fluorophores within the same membrane. As membrane-incorporated dyes such as DiOC₆ and DiIC₁₈ do not translocate from one vesicle to adjacent vesicles (Collas and Poccia, 1995c), the presence of both dyes within the same membrane is the result of a fusion event.

2.3.2. Exclusion of High Molecular Weight Dextran from Nuclei

Assembly of a continuous nuclear membrane as a result of vesicle fusion can also be monitored by the exclusion of a 150-kDa FITC-labeled dextran from nuclei (Cameron and Poccia, 1994; Collas and Poccia, 1995c; Newmeyer *et al.*, 1986; Newport and Dunphy, 1992). Nuclei without membranes or with bound but unfused membranes are permeable to the dextran (Figure 4A), whereas continuous membranes exclude the dextran (Figure 4B).

2.3.3. Electron Microscopic Assays to Monitor Nuclear Vesicle Fusion

Initially, morphological aspects of NE assembly were examined by TEM of cultured pig embryo kidney cells (Zatsepina *et al.*, 1982). These observations revealed that partially decondensed chromosomes were a preferred target for nuclear vesicles. Direct evidence for nuclear vesicle fusion and sealing of the nuclear membranes has been provided by phase contrast

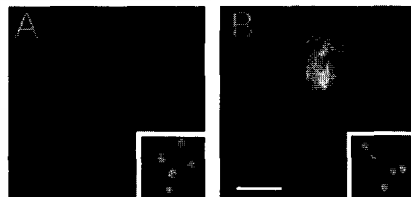


FIGURE 4. Exclusion of a 150-kDa FITC-conjugated dextran from nuclei with a continuous NE. (A) Nuclei with bound vesicles only (not shown here) do not exclude the FITC-dextran. (B) In contrast, nuclei with a continuous envelope (fused vesicles) are impermeable to the dextran. Bar, 5 μ m. Data taken from Collas and Poccia (1996a) with permission.

microscopy in *Xenopus* and *Drosophila* (Berrios and Avilion, 1990; Boman *et al.*, 1992; Vigers and Lohka, 1991; Vigers and Lohka, 1992; Wiese *et al.*, 1997; Wilson and Newport, 1988), where the nuclear membrane appears as a smooth continuous line around the chromatin periphery (Figure 5). However, nuclear vesicle fusion has been more extensively characterized in cell-free systems using TEM (Berrios and Avilion, 1990; Bornan *et al.*, 1992; Collas and Poccia, 1995c; Collas and Poccia, 1998; Nakagawa *et al.*, 1989; Vigers and Lohka, 1991; Vigers and Lohka, 1992). An example of TEM examination of nuclear vesicle binding and fusion around decondensed sea urchin sperm chromatin in cytoplasmic extract is shown in Figure 6.

Whereas the formation of an intact NE can be monitored by phase contrast microscopy or TEM, these techniques do not allow the visualization of individual vesicle fusion events. In contrast to light microscopy and TEM, field emission in-lens scanning EM (FEISEM) can inherently provide three-dimensional images of nuclear reconstruction, at a level of resolution of a few nanometers. The utility of FEISEM to investigate NE formation and NPC assembly (Goldberg and Allen, 1995; Goldberg *et al.*, 1997; Wiese *et al.*, 1997) has been demonstrated. More recently, NE assembly in *Xenopus* egg extracts has been re-examined in detail using FEISEM as an assay for the various stages of NE assembly (Wiese *et al.*, 1997). This assay led to the identification of a post-fusion "envelope smoothing" event (Figure 7).

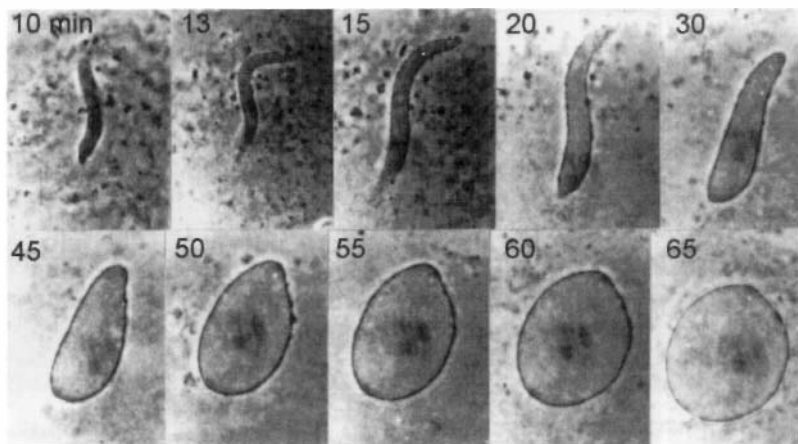


FIGURE 5. Monitoring of nuclear vesicle fusion by phase contrast microscopy. Permeabilized *Xenopus* sperm nuclei were incubated in egg cytosolic extract together with membranes as described in Wiese *et al.* (1997) and photographed at indicated time points. Taken from Wiese *et al.* (1997) with permission.

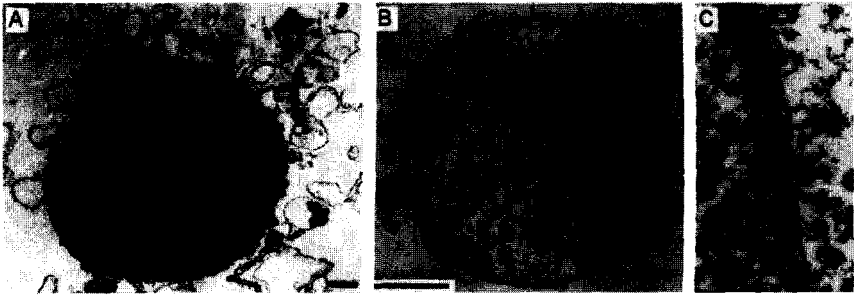


FIGURE 6. TEM assessment of nuclear vesicle binding and fusion around sea urchin sperm chromatin *in vitro*. (A) Binding of nuclear vesicles to the chromatin surface. (B) NE resulting from fusion of the chromatin-bound vesicles. Arrows in the right panel point to newly assembled nuclear pores. Bars, (A) 0.5 μm , (B) 2 μm , (C) 0.1 μm . Taken from Collas and Poccia (1995a [A]; 1998 [B]) with permission.

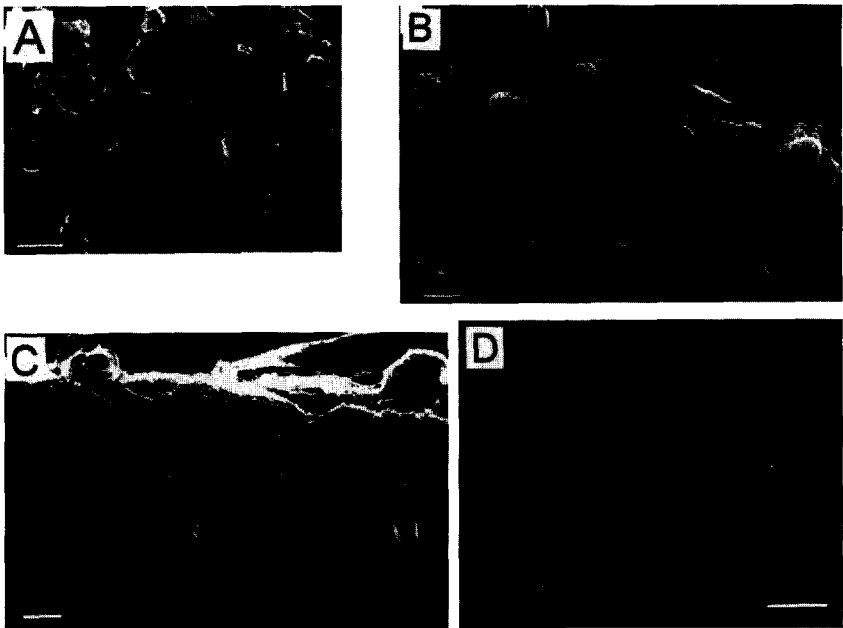


FIGURE 7. FEISEM analysis of NE formation in a *Xenopus* nuclear assembly reaction *in vitro*. (A) Nuclear vesicles bound to the chromatin surface. (B) Fusion of chromatin bound vesicles (vesicles get larger; compare with A). (C) Extensive vesicle fusion. The chromatin is enclosed by the NE which has already “smoothed” at this stage. Note the presence of many nuclear pores. (D) Low magnification view of a completely enclosed decondensed nucleus. Bar in A,B,C, 333 nm. Bar in D, 3.33 μm . Taken from Wiese *et al.* (1997) with permission.

2.4. Cytosolic and Nucleotide Requirements for Nuclear Vesicle Fusion

Cytosolic and nucleotide requirements for nuclear vesicle fusion appear to vary between systems investigated. In *Xenopus*, nuclear vesicle fusion per se does not require cytosol: both ATP and GTP together stimulate a limited amount of fusion of chromatin-bound nuclear vesicles in buffer (Boman *et al.*, 1992). Recent re-examination of nucleotide requirements for nuclear vesicle fusion in Katherine Wilson's laboratory (John Hopkins School of Medicine, Baltimore, MD) indicate that fusion of chromatin-bound vesicles takes place to the same extent with GTP alone, but not with GTP γ S, GMP-PNP, GDP, ATP or ATP γ S (Wiese *et al.*, manuscript submitted for publication), indicating a clear requirement for GTP hydrolysis, but not cytosol, for nuclear vesicle fusion. There is however an absolute additional requirement for a cytosolic fraction to promote extensive nuclear vesicle fusion resulting in a sealed NE, NE growth and NPC assembly (Boman *et al.*, 1992). In the sea urchin, nuclear vesicle fusion is also promoted by GTP, but not by GTP γ S or GMP-PNP or any other nucleotide alone. In contrast to the *Xenopus* system however, fusion also requires ATP and cytosol (Collas and Poccia, 1999a; Collas and Poccia, 1996b). A careful analysis of individual nuclear vesicle fusion as that carried out in *Xenopus* (Wiese *et al.*, 1997) has not been done in the sea urchin. Thus it remains possible that distinct requirements exist to promote limited vesicle-vesicle fusion and extensive fusion to sea urchin nuclear membranes. Nuclear vesicle fusion is also promoted by GTP in *Drosophila* extracts (Ulitzur *et al.*, 1997). These studies argue for a general requirement for GTP hydrolysis for nuclear vesicle fusion *in vitro*. In *Xenopus*, both membrane-associated and soluble GTPases are crucial for NE assembly, whereas the presence of an essential vesicle-associated GTPase in other systems remains to be shown.

GTP also stimulates fusion between outer membranes of isolated mammalian nuclei (Paient, 1981; Paient, 1984a). Furthermore, physiological concentrations of GTP also promotes fusion of purified nuclei with ER-derived microsomes stripped of ribosomes, to form large membrane extensions (Paient, 1984b). However, GTP does not stimulate fusion between other intracellular membranes (mitochondrial or Golgi) or between such membranes and nuclear membranes (Paient, 1984b). Supporting this view, we also found that the Golgi-derived sea urchin egg vesicle fraction MV2 α by itself (see Table 1) is incapable of fusing with sperm LSs or any other egg membrane fraction (Collas and Poccia, 1996b), nor with already assembled NEs (P.C., unpublished). Fusion of MV2 α with LSs, however, requires the fusogenic fraction MV1 (Collas and Poccia, 1996b) (see below). In cell-free ER assembly assays, cytosol containing ATP,

GTP and an ATP-generating system also stimulates fusion of ER-derived rat liver microsomes with each other (Lavoie *et al.*, 1996). Replacement of GTP with GTP γ S or other analogues inhibited microsome fusion. Notably, GTP alone induced rough (but not smooth) microsome fusion in the absence of cytosol (Lavoie *et al.*, 1996). In contrast to these studies, however, there is no GTP-requirement for ER membrane fusion in the yeast *in vitro* system, and homotypic ER-ER fusion is not inhibited by GTP γ S (Latterich and Schekman, 1994). Nevertheless, ER membrane fusion in yeast requires ATP (Latterich and Schekman, 1994). The results from these studies argue that there might be an organelle specificity of nucleotides capable of stimulation fusion, suggesting that fusion is regulated by membrane composition.

3. INVOLVEMENT OF SMALL GTP-BINDING PROTEINS IN NUCLEAR VESICLE DYNAMICS

3.1. Nuclear Vesicle Fusion Requires GTP Hydrolysis

Nuclear vesicle fusion is potently inhibited by the GTP analogue GTP γ S, when applied instead of, or together with, GTP (Wiese *et al.*, manuscript submitted for publication). This strongly suggests that fusion requires GTPase activity (Boman *et al.*, 1992; Collas and Poccia, 1995c; Macaulay and Forbes, 1996; Newport and Dunphy, 1992). GTP-mediated fusion of nucleotide-stripped vesicles also takes place in buffer, thus the GTPase(s) involved must be membrane-associated. In addition, because cytosol is required for extensive fusion and NE formation, there might also be a requirement for soluble GTPase activity. A number of experiments, primarily involving *Xenopus* nuclear assembly cell-free reactions, have addressed the requirement and origin of putative GTP binding proteins that stimulate nuclear vesicle fusion. There seems to be an organelle specificity of nucleotides capable of stimulating fusion, suggesting that membrane composition regulates this process.

3.2. Early Evidence for a Putative Role of ARFs in Nuclear Vesicle Dynamics

ADP-ribosylation factors (ARFs) are members of a family of ~21-kDa GTP-binding proteins implicated in cellular membrane traffic such as ER to Golgi transport, intercisternal Golgi transport and exocytic vesicle traffic (see Spiro *et al.*, 1995). ARF activity is probably regulated by guanine nucleotide exchange and hydrolysis since cytosolic ARF is in a GDP-bound

form while GTP-bound ARF binds to membranes (Kahn, 1991). Biochemical and immunofluorescence analyses have shown that ARF proteins are primarily associated with the Golgi, but are also present on clathrin-coated and non-clathrin-vesicles in the presence of GTP γ S (Gant and Wilson, 1997). ARFs have been proposed to regulate coat assembly (Ostermann *et al.*, 1993), although the role of the GTP-binding activity of ARF in this process remains unclear.

Inhibition of nuclear vesicle fusion with GTP γ S was initially found to be mediated by a soluble factor named GSF (for GTP- or GTP γ S-dependent soluble factor), which associates with vesicles treated with GTP γ S, and inhibits fusion of these vesicles in NE assembly reactions lacking GTP γ S (Boman *et al.*, 1992). GSF was proposed to be a member of the ARF family of small GTPases because vesicles preincubated with purified bovine ARF1 and GTP γ S did not fuse in reactions lacking GTP γ S (Boman *et al.*, 1992), and purification of soluble GSF activity from *Xenopus* egg cytosol identified ARFs as the most likely the only soluble proteins with GSF activity (Boman *et al.*, 1996).

3.3. Evidence for a Non-ARF GTPase Active in Nuclear Envelope Assembly

Re-examination of the putative role of ARF in inhibition of nuclear vesicle fusion mediated by GTP γ S revealed that ARF is in fact not required for nuclear vesicle fusion (Gant and Wilson, 1997). In *Xenopus* egg cytosol depleted of ARF by size-fractionation, nuclear vesicles bind and fuse to form nuclear membranes. Furthermore, brefeldin A (BFA), which blocks the nucleotide exchange necessary to activate ARF and inhibits ARF binding to membranes, does not prevent nuclear assembly. Thus, ARF does not seem to be essential for NE formation *in vitro*. Nevertheless, nuclear vesicle fusion remains inhibited by GTP γ S in ARF-depleted cytosol and in cytosol containing BFA.

In support of the absence of ARF function in nuclear vesicle dynamics, other intracellular vesicle fusion events are also ARF-independent. Despite the original idea that ARF was involved in endosome-endosome fusion *in vitro*, based on inhibition of fusion with GTP γ S and restoration with purified ARF1 (Lenhard *et al.*, 1992), endosome-endosome fusion kinetics are not altered in ARF-depleted cytosol or with BFA (Spiro *et al.*, 1995). However, ARF-depleted cytosol fails to support inhibition of fusion by GTP γ S, whereas this inhibition is restored by addition of purified ARF1 (Spiro *et al.*, 1995). This argues that soluble ARFs are not required for endosomal vesicle fusion *in vitro*, but remain responsible for inhibition of fusion in the presence of GTP γ S and cytosol.

There is a possibility that another soluble GTPase acting similarly to ARF inhibits nuclear vesicle fusion in the presence of GTP γ S. However, identification of ARF as the most probable soluble component capable of inhibiting fusion of GTP γ S-treated membranes (Boman *et al.*, 1996), most likely rules out this hypothesis. Alternatively, another GTPase(s) may be required for nuclear vesicle fusion. This GTPase may be soluble, as suggested by the cytosolic requirement for extensive nuclear vesicle fusion, and detectable cytosolic GTPase activity (Wiese *et al.*, manuscript submitted for publication). The same study predicts the existence of a membrane-associated GTPase which stimulates nuclear vesicle fusion in the absence of cytosol. Association of this GTPase with the membrane is salt-resistant, but whether it is an integral membrane protein or a strongly anchored peripheral protein is at present unknown. Interestingly, this GTPase is believed not to be ARF, as salt-treated vesicles lack detectable ARF, yet still undergo fusion. It will be of interest to investigate how the membrane-associated GTPase, putative additional soluble GTPase(s) and/or other soluble components co-operatively and independently function in nuclear vesicle fusion.

4. ANALOGIES BETWEEN NUCLEAR VESICLE FUSION AND FUSION EVENTS IN INTRACELLULAR MEMBRANE TRAFFICKING

4.1. Inhibition of Nuclear Vesicle Fusion with the Sulphydryl Modifier, *N*-Ethylmaleimide

The sulphydryl alkylating agent, *N*-ethylmaleimide (NEM) has been known for several years to have adverse effects on NE assembly. Pre-treatment of nuclear vesicles with millimolar concentrations of NEM, followed by quenching of excess NEM with dithiothreitol, does not affect the ability of vesicles to bind chromatin but potently inhibits fusion (Collas and Poccia, 1996b; Macaulay and Forbes, 1996; Newport and Dunphy, 1992; Vigers and Lohka, 1991). As a result, formation of a double nuclear membrane and assembly of NPCs are prevented (Macaulay and Forbes, 1996). NEM does not affect post-fusion events in the NE assembly process such as vesicle flattening or the assembly of NPCs once the membranes have fused (Macaulay and Forbes, 1996). The ability of the sea urchin fusogenic vesicle fraction MV1 to bind LSs is also sensitive to NEM treatment of the vesicles (Collas and Poccia, 1996b). Treatment of LS-bound MV1 vesicles with NEM also affects LS-MV1 fusion (P.C., unpublished data). The sensi-

tivity of nuclear vesicle fusion to NEM is consistent with the possibility that NEM-sensitive factors such as NSF, SNAPs or p97 are involved in nuclear vesicle fusion during NE assembly.

4.2. Targeted Membrane Fusion Orchestrated by Components of the SNARE Hypothesis

The SNARE hypothesis (Rothman and Warren, 1994) predicts that soluble NSF attachment protein (SNAP) receptors on a vesicle membrane (v-SNAREs) bind to SNAP receptor proteins on target membranes (t-SNAREs) in a process known as vesicle docking (binding). Fusion of the docked vesicle with the target membrane is stimulated by binding of the soluble components NEM-sensitive factor (NSF) and α -SNAP to the SNARE complex. Fusion is driven by the ATPase activity of NSF. Members of the v- and t-SNARE families are implicated in specific steps of vesicular trafficking in mammalian and yeast cells; thus the specificity of vesicular transport may be determined by SNAREs. Recently, the surprising finding that v- and t-SNAREs reconstituted into separate lipid bilayer vesicles assemble into SNARE-SNARE complexes connecting the two vesicles, are sufficient to elicit fusion of these vesicles, implies that "SNARE-pins" constitute the minimal machinery for intracellular vesicle fusion (Weber *et al.*, 1998). Several excellent reviews on the control of intracellular membrane fusion report recent advances in this area (Götte and Fischer von Mollard, 1998; Rothman and Sollner, 1997; Rothman and Warren, 1994; Sollner, 1995).

4.3. A Role for p97 in Nuclear Envelope Assembly?

Cytoplasmic membrane fusion events are catalyzed by at least two soluble ATPases, including NSF, identified as the NEM-sensitive factor required for intra-Golgi vesicle transport and other vesicle-mediated trafficking steps (Rabouille *et al.*, 1998). Another ATPase, p97, is highly homologous to NSF (Peters *et al.*, 1990). The putative relevance of p97 in nuclear vesicle fusion is illustrated by its role in the fusion of outer nuclear membranes during yeast karyogamy (Latterich *et al.*, 1995) and in Golgi reassembly under mitotic conditions (Rabouille *et al.*, 1995).

In the yeast *S. cerevisiae*, nuclear and ER membranes remain intact throughout mitotic division. However, fission of the NE, which is continuous with the ER, involves at least one fusion step to generate daughter nuclei. In addition, during cell mating, parental nuclei fuse at the level of the nuclear membranes to form a diploid nucleus (Latterich and Schekman,

1994). Using a cell-free assay that reproduces fusion of ER-nuclear membranes in yeast, it was shown that the *CDC48* gene product, a homologue of p97 (Peters *et al.*, 1990) was necessary for ER membranes to fuse homotypically (Latterich *et al.*, 1995). Such fusion, however, does not require NSF or a-SNAP (Latterich *et al.*, 1995). Thus, p97 is clearly implicated in fusion events at the level of the nuclear membrane.

p97 also functions in reconstitution of the Golgi from mitotic rat liver Golgi fragments *in vitro* (Rabouille *et al.*, 1998; Rabouille *et al.*, 1995). Evidence to support this comes from the observation that purified p97 restores growth of Golgi cisternae after inactivation of Golgi vesicles with NEM (Rabouille *et al.*, 1995). We have reported that a sub-fraction of nuclear vesicles contains a Golgi marker enzyme (sea urchin MV2 α) and that MV2 α vesicles fuse with themselves (homotypic fusion) and with those of MV1 and MV2 β (qualifying as heterotypic fusion). Since p97 has been suggested to be implicated in homotypic intracellular fusion events (Rabouille *et al.*, 1995), p97 or a p97-homologue may contribute to homotypic MV2 α -MV2 α fusion during male pronuclear envelope formation. It is also not excluded that p97 functions in heterotypic fusion events (as NSF does) and might be involved in fusion of Golgi-derived vesicles with vesicles originating from the ER or other sources to assemble nuclear membranes.

Together with the NEM-inhibition of nuclear vesicle fusion in several systems, the above observations are consistent with, but do not demonstrate, an involvement of p97 in nuclear vesicle fusion. Cytosol and membrane fractionation experiments, and functional inhibition studies with antibodies to soluble ATPases involved in intracellular vesicle fusion may provide some insight on the nature of the NEM-sensitive factors involved in NE assembly.

4.4. Implication of SNAREs in Nuclear Vesicle Targeting and Fusion: An Argument

The SNARE hypothesis initially predicted that individual v- and t-SNAREs would provide specificity of vesicle targeting in intracellular trafficking. Recent studies of SNARE function in yeast however, suggest that this specificity may not be as narrow as initially thought. Results show that a t-SNARE can define a target membrane and interact with multiple V-SNAREs on different incoming vesicle populations (see Götte and Fischer von Mollard, 1998). The involvement of the yeast *cis*-Golgi t-SNARE Sed5p in both anterograde and retrograde traffic to the *cis*-Golgi compartment (Götte and Fischer von Mollard, 1998) supports a view of SNARE-mediated multidirectional and complex vesicle targeting and fusion events.

Consistent with these hypotheses is the high specificity of targeting of sea urchin MV1 and MV2 α vesicle to the LSs, even in large excess of such vesicles in the binding reaction (Collas and Poccia, 1996b). Thus, it is conceivable that targeting of multiple nuclear vesicle populations to LSs may represent an LS t-SNARE-mediated targeting and docking of several distinct, but selective, V-SNAREs on the vesicles. Interactions of chromatin bound vesicles with additional incoming vesicles may be regulated by a similar pathway.

5. A ROLE OF NUCLEAR Ca^{2+} IN NUCLEAR VESICLE FUSION?

5.1. A Ca^{2+} Store at the Nuclear Envelope

The inner and outer nuclear membranes are separated by a lumen that is continuous with the ER lumen. As in the ER, this luminal space acts as a store of compartmentalized free Ca^{2+} . The NE harbors an ER-type Ca^{2+} pump which concentrates Ca^{2+} into the NE lumen, functional receptors for the Ca^{2+} -releasing messenger inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors (Gerasimenko *et al.*, 1995; Humbert *et al.*, 1996). Each type of receptor is localized both in the inner and outer nuclear membrane (Gerasimenko *et al.*, 1995; Humbert *et al.*, 1996; Malviya and Rogue, 1998; Santella and Carafoli, 1997). The NE also contains mechanisms of IP_3 production (Divecha *et al.*, 1993). Upon appropriate signalling, IP_3 binds to the NE IP_3 receptors causing Ca^{2+} channels to open, and release Ca^{2+} into the cytosol and the nucleoplasm (Berridge and Irvine, 1984; Gerasimenko *et al.*, 1995; Humbert *et al.*, 1996).

5.2. Generating Ca^{2+} Signals in the Nucleus

The localization of IP_3 and ryanodine receptors at the NE, although it may not be exclusive, argues in favor an autonomous regulation of nuclear Ca^{2+} signals (Malviya and Rogue, 1998). IP_3 or cADP ribose binding to their receptors on the inner nuclear membrane are anticipated to open second-messenger-regulated Ca^{2+} release channels, propagating Ca^{2+} signals into the nucleoplasm (Malviya and Rogue, 1998). Nuclei also contain systems to generate IP_3 (Divecha *et al.*, 1993), which, in the cytoplasm, is essential to generate Ca^{2+} oscillations. Refilling of nuclear Ca^{2+} pools occurs via an ATP-mediated nuclear Ca^{2+} transporter localized on the outer nuclear membrane (Humbert *et al.*, 1996) and stimulated by CAMP-dependent phosphorylation (Malviya and Rogue, 1998). Other pathways for

replenishing nuclear Ca^{2+} stores are also under current investigation (Malviya and Rogue, 1998).

In addition to the membrane-associated Ca^{2+} pumps, the NPC is probably essential for the transport of Ca^{2+} in and out of the nucleus. Permeability of the NPC is affected by the Ca^{2+} concentration in the NE lumen (Perez-Terzic *et al.*, 1996). Recent observations in Ueli Aebi's laboratory (University of Basel, Switzerland) also point to a direct regulation of NPC permeability by Ca^{2+} , as evidenced by specific and reversible Ca^{2+} -mediated opening and closing of the NPC (Malviya and Rogue, 1998). To support these views, both signal-mediated import of karyophilic proteins and passive diffusion of smaller molecules (Greber and Gerace, 1995; Stehno-Bittel *et al.*, 1995) into the nucleus are inhibited when living cells or oocyte nuclei are treated with ionophores to deplete luminal Ca^{2+} .

5.3. Evidence for Nuclear Ca^{2+} Independent Nuclear Envelope Assembly

A series of experiments initially suggested that Ca^{2+} was implicated in nuclear assembly *in vitro*. Chelation of free cytosolic Ca^{2+} with BAPTA was shown to inhibit at least two distinct steps of NE assembly in *Xenopus* egg extracts, namely nuclear vesicle fusion (Sullivan *et al.*, 1993) and NPC assembly (Macaulay and Forbes, 1996). It has been proposed that BAPTA inhibits NE assembly by suppressing cytosolic Ca^{2+} gradients originating from local releases of Ca^{2+} (Sullivan *et al.*, 1993). Consistent with this hypothesis, antibodies against type 1 IP_3 receptor and heparin, a potent competitive inhibitor of the IP_3 receptor, were shown to inhibit nuclear assembly *in vitro* (Sullivan *et al.*, 1995; Sullivan *et al.*, 1993). These observations raised the possibility that activation of IP_3 receptors might be necessary for NE assembly (Sullivan and Wilson, 1994).

More recent studies have re-examined in detail the putative role of luminal nuclear Ca^{2+} in NE assembly. Depletion of luminal Ca^{2+} pools by pre-treatment with Ca^{2+} ionophores or selective inhibitors of the Ca^{2+} -sequestering pumps had no effect on the assembly of nuclei with fused and sealed nuclear membranes and functional nuclear pores (Marshall *et al.*, 1997a). These results argue that deficient nuclear import observed in ionophore-treated living cells (Greber and Gerace, 1995) is not directly due to the depletion of luminal Ca^{2+} stores. The latter findings of Marshall *et al.* (1997) may be reconciled with earlier reports on the effect of Ca^{2+} on nuclear assembly in that the effect of BAPTA on nuclear vesicle fusion and NE assembly may be due to chelation of Zn^{2+} , rather than Ca^{2+} . The recent finding that TPEN, a potent $\text{Zn}^{2+}/\text{Fe}^{2+}$ chelator with low affinity for Ca^{2+} , inhibits lamina assembly and destabilizes nuclear architecture supports this

view (Shumaker *et al.*, 1998). Similarly, it is possible that IP3 receptors play a role in nuclear vesicle fusion that is independent of their Ca^{2+} releasing property.

6. NUCLEAR VESICLE FUSION REQUIRES MEMBRANE-ASSOCIATED FUSIGENIC ELEMENTS

6.1. Proteins Mediating Nuclear Membrane Fusion in Yeast Are Being Identified

In addition to putative SNAREs (yet to be identified) of nuclear vesicles, a number of membrane proteins may also be directly involved in fusion. To our knowledge, no such proteins have been identified to date in the organisms most commonly used in nuclear assembly assays. However, candidates for membrane proteins implicated in membrane recognition and fusion have been identified in a genetic screen for karyogamy mutants in *S. cerevisiae* (Kurihara *et al.*, 1994). These results and others (Beh *et al.*, 1997) argue that ER homotypic fusion events mediate NE fusion (karyogamy) when two yeast cells mate. Mutations in several *KAR* genes disrupt a late stage of karyogamy that corresponds to the membrane recognition or fusion step. Based on cytological and genetic criteria, all *KAR* mutants have been grouped into two functional classes. Class I mutants, defined by the genes *KAR1*, *KAR3*, *KAR4*, exhibit a block in nuclear apposition, where nuclei remain distant from one another and do not fuse. Class II mutants, defined by the genes *KAR2*, *KAR5*, *KAR7* and *KAR8*, display closely juxtaposed but unfused nuclei (Beh *et al.*, 1997). Disruption of nuclear membrane fusion by these genes has been documented both *in vivo* (Kurihara *et al.*, 1994) and in cell-free ER-nuclear membrane fusion assays referred to earlier (Beh *et al.*, 1997; Kurihara *et al.*, 1994; Latterich and Schekman, 1994).

A well characterized class II gene is *KAR2*, which encodes an ER luminal HSP70. The protein Kar2p is a yeast homologue of mammalian proteins BiP. Kar2p has been proposed to have different roles in nuclear membrane fusion (Beh *et al.*, 1997). It may be indirectly required for nuclear fusion via a function in translocation and/or folding of essential components of the fusion machinery, or may be necessary to maintain NE structure during fusion. It appears more likely, however, that Kar2p is directly involved in nuclear fusion, by acting as a molecular chaperone contributing to the assembly of a fusogenic complex (Beh *et al.*, 1997).

Kar5p, the product of the *KAR5* gene, has been recently characterized and also found to play a direct role in nuclear fusion (Beh *et al.*, 1997). Kar5p is induced as part of the pheromone response pathway, and

interestingly, is localized near the initial site of membrane fusion. Because a portion of Kar5p extends into the lumen of the EWE, with virtually no cytoplasmic domain (Beh *et al.*, 1997), it is unlikely that the protein mediates membrane binding recognition or even fusion per se. Rather, it has been proposed that Kar5p is required for completion of membrane fusion (perhaps by dilating the fusion pore) after initial membrane contact has been established (Beh *et al.*, 1997).

6.2. Relevance of Kar Protein Homologues in Nuclear Vesicle Fusion

The relevance of Kar proteins or homologues for nuclear vesicle fusion during NE assembly remains an open question. Identification of fusigenic membrane populations (MV1 of sea urchin and NEP-A of *Xenopus*) suggests that elements associated with these vesicles must exist, that mediate fusion. These are likely to be proteins (Collas and Poccia, 1996a; Collas and Poccia, 1996b); however, the existence of fusigenic "signals" mediated by lipid components of the membrane is not excluded. The fractionation of cytoplasmic vesicles into distinct populations in sea urchin and mammalian systems with different binding and fusion properties should facilitate such studies and provide a clue to the mechanism of nuclear vesicle fusion. Further refinements of *in vitro* nuclear assembly systems, the emergence of genetic tools in a variety of organisms, reconstitution of recombinant proteins into liposomes, as well as the steady increase in our understanding of ER, Golgi and endosome vesicle fusion processes should provide valuable answers.

ACKNOWLEDGMENTS. The authors are thankful to Dr. Katherine L. Wilson (Johns Hopkins University School of Medicine, Baltimore, MD) for sharing data before publication (referred to as "Wiese *et al.*, manuscript submitted for publication" in the text). Dr. Jean-Claude Courvalin (CNRS, Institut Jacques Monod, Paris, France) is thanked for numerous discussion and continuous access to this laboratory. Work of the authors was supported by NSF Grants DCB 9004170 and IBN-9304394 to D.L.P., an Amherst College Faculty Research Award to DLP, and Grant A99/111 from the Norwegian Cancer Society to P.C. P.C. is a Norwegian Cancer Society Fellow.

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Chapter 8

Transactions at the Peroxisomal Membrane

Ben Distel, Ineke Braakman, Ype Elgersma,*
and Henk F. Tabak

1. INTRODUCTION

Peroxisomes are small organelles of eukaryotic cells with distinct contributions to cellular metabolism. Their manifestation and enzymatic content can vary depending on cell type or organism. This diversity is reflected by the different names for the organelles: peroxisomes, glyoxysomes, glycosomes. Based on conserved features of protein import and metabolic pathways, however, they are now considered to constitute one microbody family.

They were discovered by DeDuve and co-workers during biochemical fractionation studies on rat liver homogenates as small vesicles containing catalase and a number of H_2O_2 producing enzymes such as urate oxidase, D-amino acid oxidase and α -hydroxyacid oxidase (Baudhuin *et al.*, 1965).

BEN DISTEL, INEKE BRAAKMAN, YPE ELGERSMA,* and HENK F. TABAK
Department of Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. *Present address: University of California at Los Angeles, Department of Neurobiology, Los Angeles, California 90095.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

On the basis of this initial inventory the name peroxisomes was coined to denote the organelles. Furthermore, the enzymes were not thought to have an essential function in the cell and DeDuve even considered the possibility that peroxisomes constitute “fossil organelles” (DeDuve and Bauduin, 1966).

Our attitude towards peroxisomes is totally different now. Three important developments are responsible for this change in view:

- i) In 1973 Goldfischer *et al.* reported that peroxisomes could not be detected in the cells of a patient with a severe clinical phenotype. This disease had been described before in the literature by Zellweger: the cerebro-hepato-renal or Zellweger syndrome. It underscored the importance of peroxisomal function and was the start of a still increasing list of human diseases related to peroxisome malfunction. They range from an almost total absence of the organelles to single enzyme deficiencies. The study of these diseases has been instrumental in the discovery of the various processes in human metabolism in which peroxisomes partake. These include the β -oxidation of long chain and very long chain fatty acids, the first steps in plasmalogen biosynthesis (lipid components of membranes), bile acid and cholesterol biosynthesis etc. (Lazarow and Moser, 1994; Mannaerts and Van Veldhoven, 1993; Van den Bosch *et al.*, 1992). Apparently, the first enzymes discovered by DeDuve *et al.* with the then available enzymatic assays, were an unfortunately unrepresentative lot.
- ii) Microbodies had a much better start in the research on other kingdoms of nature since clear and essential metabolic pathways were confined to the organelles. For instance, the enzymes of the glyoxylate cycle which allow fungi, protozoa, nematodes and plants to convert fat into carbohydrate, largely reside in microbodies called glyoxysomes (Beevers, 1969). Certain fungi that are specialized to grow on alcohols, alkanes or fatty acids, harbour the necessary enzymes in their microbodies. This immediately illustrates how important microbodies are to these organisms, but also demonstrates the versatility and specialization of microbodies. It makes it understandable that the unity among the microbody family was not appreciated immediately after their discovery.
- iii) In recent years various yeasts, *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris* and *Yarrowia lipolytica* have been used for a genetic approach to study the various processes involved in the maintenance, biosynthesis and prolifer-

ation of peroxisomes (Subramani, 1998; Elgersma and Tabak, 1996a). This has created a wealth of new information, experimental tools and mutants that can serve as models to study the various human diseases.

2. THE ISOLATION OF YEAST MUTANTS DISTURBED IN PEROXISOME FUNCTION

A significant increase in our knowledge about the biogenesis of peroxisomes came from the genetic approach using a variety of yeasts. In all these yeast species the peroxisomal compartment can enormously vary in volume and number depending on growth conditions. Because of glucose repression only a few small peroxisomes are present when cells are grown in high concentrations of glucose. Growth on non-fermentable carbon sources leads to a modest increase in the peroxisomal compartment but a spectacular increase is seen on carbon sources that require peroxisomal function such as certain alcohols, alkanes or fatty acids. For instance, when *H. polymorpha* is grown in a chemostat on a limiting amount of methanol, more than 90% of the cellular volume is occupied by large cubic sized microbodies (Veenhuis *et al.*, 1978). This versatility to switch from conditions in which peroxisomes are not essential to conditions in which they are absolutely required for growth on specific carbon sources, has been exploited for the isolation of mutants disturbed in peroxisome function.

Although all these yeasts, except *S. cerevisiae* are well adapted to growth on specific carbon sources, they were less suitable for the application of refined genetics and recombinant DNA techniques. Initially, this may have been a handicap but especially *P. pastoris* and *H. polymorpha* quickly made up their arrears. On the other hand the fatty acid oleate was discovered to induce peroxisome proliferation in *S. cerevisiae* (Veenhuis *et al.*, 1987). The generation time on this substrate is long, however, and the growth conditions compare unfavorably compared to the other yeasts. This disadvantage is outweighed by the advanced genetics and manipulations *S. cerevisiae* allows and the wealth of information already gathered for this organism. The genome project has been finished, generating many new ORFs (Goffeau *et al.*, 1996), some of which turn out to be highly relevant to peroxisome biogenesis.

The first mutants unable to grow on oleate (Oleate Non-Utilizers or ONUs') were isolated in *S. cerevisiae* (Erdmann *et al.*, 1989). Among these were *pas* mutants (according to new nomenclature rules now called *pex* mutants (Distel *et al.*, 1996)) in which (some) peroxisomal marker enzymes

remained in the cytoplasm and (normal) peroxisomes could not be observed based on biochemical fractionation and electron microscopical experiments. These *pex* mutants were of great interest because they suggest defects in genes whose products play a direct role in the biogenesis of the organelles. The *pex* mutants are considered to be good models for the human diseases in which a similar loss of peroxisomal structures has been observed. Subsequently, positive genetic screening tests have been introduced to isolate more *pex* mutants (Lazarow, 1993; Zhang *et al.*, 1993; Elgersma *et al.*, 1993; Van der Leij *et al.*, 1992).

The excellent growth properties on one or more specific carbon sources has made it much easier to obtain a collection of mutants for *H. polymorpha* and *P. pastoris*, and the list of *pex* mutants is growing rapidly. At the moment a list of 21 *pex* mutants is available (an updated list can be viewed on the following web site: www.mips.biochem.mpg.de/proj/yeast/reviews/pex_table.html).

The mutants were used for the cloning of the corresponding wild-type genes (*PEX*) by functional complementation. In some cases intriguing gene products (called peroxins) have been identified with clear homologies to known protein families but relating them to biological function is still difficult.

3. IMPERMEABILITY OF THE PEROXISOMAL MEMBRANE

Carefully isolated mitochondria show latency: exogenously added substrates can only reach the matrix-located enzymes when the mitochondrial inner membrane is perturbed by, for instance, the addition of detergent. Despite similar care during isolation, peroxisomes did not show such latency and molecules like sucrose could freely traverse the peroxisomal membrane (VanVeldhoven *et al.*, 1983). In more extreme cases even matrix-located enzymes leaked out which is reason to still debate the location of enzymes participating in the glyoxylate cycle (McCammon *et al.*, 1990).

The permeability was suggested to be due to the existence of integral membrane proteins resembling mitochondrial porins, which allow small molecules to cross the outer membrane. This notion was supported by patch-clamp studies carried out on membranes taken from a highly purified peroxisomal fraction (Van Veldhoven *et al.*, 1987). Now, however, we are convinced that the peroxisomal membrane, like most cell membranes, forms a closed barrier to even small molecules. The possibility to grow *H. polymorpha* cells that consist for more than 90% of peroxisomes allowed *in vivo* nuclear magnetic resonance measurements to derive the internal pH of peroxisomes (Nicolay *et al.*, 1987). It is somewhat more acidic than the

pH of the cytosol (pH 5.8–6 versus pH 7) implying that protons cannot leave the peroxisome. Using specifically engineered mutants in combination with biochemical assays we have made it plausible that metabolites like NADH, NADP and acetyl-coA cannot diffuse out of peroxisomes, suggesting the existence of metabolite transporters (Elgersma and Tabak, 1996a; Van Roermund *et al.*, 1995; Elgersma *et al.*, 1995). A large set of transporters mediating metabolite transport across the mitochondrial inner membrane already has been described. Such information is almost completely lacking for peroxisomes. The first clue for a peroxisomal transporter came from patient studies in man. Young boys that suffer from a disease called X-linked adrenoleukodystrophy (X-ALD) displayed an elevated concentration of long-chain fatty acids in body fluids. The affected gene was identified by positional cloning and the encoded protein turned out to be an ABC (ATP Binding Cassette) half-transporter family member residing in the peroxisomal membrane (Mosser *et al.*, 1994; Mosser *et al.*, 1993). When orthologs of this protein showed up during the yeast genome sequence project (Bossier *et al.*, 1994), we and others studied the function of these peroxisomal ABC transporters in yeast (Hettema *et al.*, 1996; Shani *et al.*, 1996). The data suggest that two ABC half-transporters (Pat1p/Pat2p) combine into a functional complex which can import activated long-chain fatty acids from the cytosol into peroxisomes. Since all members of the ABC integral membrane protein family in one way or another are involved in transport of molecules across membranes, their existence in peroxisomal membranes is strong support for the notion that the peroxisomal membrane is impermeable to small molecules *in vivo*. A candidate for a second transporter is Pex11p (vide infra) and there are likely to be more.

4. IMPORT OF PROTEINS INTO PEROXISOMES

Growth and maintenance of the peroxisomal compartment requires the specific targeting of proteins and lipid components to the organelles. The current view is that proteins are synthesized in the cytoplasm, folded to a large extent and subsequently translocated across or inserted into the peroxisomal membrane. There are only vague ideas how integral membrane proteins reach the peroxisome. It probably requires a different set of proteins, like in mitochondria (Adam *et al.*, 1999; Köhler *et al.*, 1998) because it is not dependent on components of the pathway responsible for the import of matrix proteins. Possible candidates under investigation are Pex3p, Pex16p and Pex19p (South and Gould, 1999; Götte *et al.*, 1998; Baerends *et al.*, 1996; Wiemer *et al.*, 1996). Comparison of primary amino acid sequences of membrane proteins has pinpointed consensus motifs that

might be used for targeting of these proteins to peroxisomes (Dyer *et al.*, 1996). Even less is known of how lipids are recruited to the membranes of enlarging peroxisomes.

Major progress in understanding protein targeting has been made especially for peroxisomal matrix proteins. Serendipity was very helpful when it was observed that the well-known reporter protein luciferase, expressed in African Green Monkey cells, showed punctate fluorescence coinciding with peroxisomes. Indeed, it was shown that luciferase is a peroxisomal matrix protein in the lantern organ of fireflies (Keller *et al.*, 1987). The targeting signal is a tripeptide SKL located at the C-terminal end of luciferase (Gould *et al.*, 1987). At the moment a lot of peroxisomal matrix proteins are known that have this peroxisomal targeting sequence (PTS1) or a conserved derivative thereof (Elgersma *et al.*, 1996c; Gould *et al.*, 1989). Later, a different, N-terminally located sequence was discovered that is responsible for import of thiolase into peroxisomes (PTS2) (Osumi *et al.*, 1991; Swinkels *et al.*, 1991). There is only a limited number of proteins that follow the PTS2 pathway.

The existence of two import signals suggests the presence of specialized components of the import machinery to accommodate the import of both PTS1 and PTS2 containing proteins. This is borne out by the occurrence of two *S. cerevisiae* mutants: *pex5* and *pex7* (Van der Leij *et al.*, 1992). Pex5p and Pex7p are soluble proteins that specifically recognise PTS1 respectively PTS2 import signals (reviewed in Erdmann *et al.*, 1997). Although there is still debate as to whether Pex7p is a cytosolic or peroxisomal protein or whether it functions at both locations (Lazarow and Kunau, 1997), a general working model is that Pex5p and Pex7p pick up their corresponding peroxisomal matrix proteins in the cytosol and deliver them to docking proteins located in the peroxisomal membrane. After delivery they cycle back to pick up their next cargo (Figure 1).

Identification of the components of the membrane-located translocation machinery turned out to be more difficult. A number of *pex* mutants are integral membrane or membrane associated proteins but it is difficult to establish whether they have a direct or indirect role in protein import. In certain cases peroxisomes showed altered morphology, ranging from still recognizable organelles to residual peroxisomal remnants ("ghosts"). In other cases such remnants are even absent. The import of PTS1 and/or PTS2 proteins or GFP derivatives is the only specific assay we can rely on to distinguish between protein import per se or other aspects of peroxisome biogenesis. An extreme example of how difficult it is to identify the correct function of a membrane protein is *PEX11*. *Pex11*, although considered a *pex* mutant, was not found in the genetic screens for the isolation of *pex* mutants. Instead, it was isolated as a major membrane constituent of

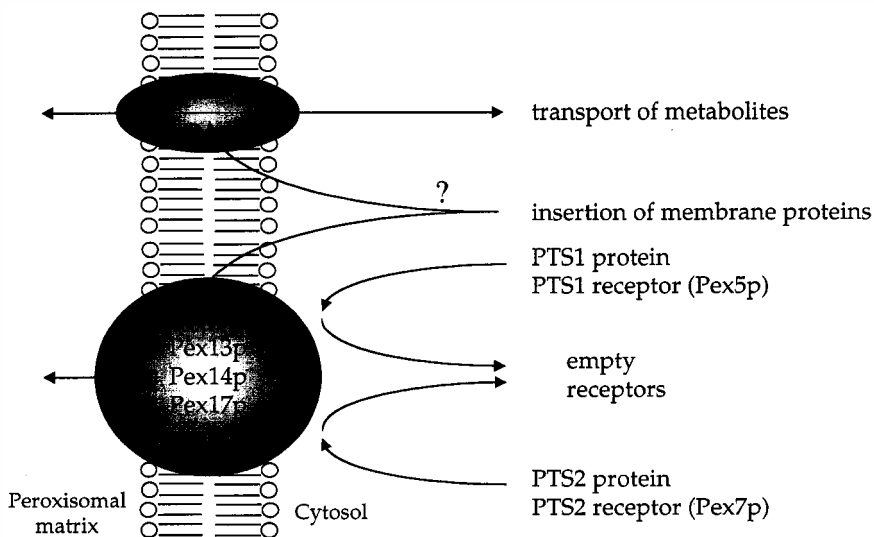


FIGURE 1. Translocation of proteins and metabolites across the peroxisomal membrane. Peroxisomal matrix and membrane proteins are targeted to the peroxisome via separate pathways. PTS containing proteins bind their cognate receptors, Pex5p and Pex7p, in the cytosol and are shuttled to the peroxisomal membrane. The membrane located docking complex for the receptors consists of at least three proteins: Pex13p, Pex14p and Pex17p of which Pex13p is the only bona fide integral membrane protein. After delivery of their cargo the receptors cycle back to the cytosol to pick up new cargo. Components required for peroxisomal membrane protein targeting and insertion have not yet been identified. Metabolite carriers are present in the peroxisomal membrane to shuttle metabolites across the membrane. For further details see text.

peroxisomal membranes and the gene was cloned after determination of part of its amino acid sequence (Marshall *et al.*, 1995; Erdmann and Blobel, 1995). The *pex11* mutant is not disturbed in the import of matrix proteins but shows enlarged peroxisomes. Overexpression of Pex11p in yeast as well as in mammalian cells resulted in large numbers of small peroxisomes (Schrader *et al.*, 1998; Erdmann and Blobel, 1995; Marshall *et al.*, 1995). Moreover, mammalian Pex11p interacted with a component of the coatamer complex (see below). These observations were interpreted to suggest a role for Pex11p in division of peroxisomes. Upon closer examination, our evidence suggests that it is involved in metabolism by transporting metabolites across the peroxisomal membrane (Hettema and Van Roermund, personal communication).

Two hybrid interaction analysis between the PTS1 and PTS2 receptor proteins and peroxisomal membrane proteins has provided the best results

in marking components of the protein import machinery (Huhse *et al.*, 1998; Albertini *et al.*, 1997; Erdmann and Blobel, 1996; Gould *et al.*, 1996; Elgersma *et al.*, 1996b). In Figure 1 these candidates, Pex13p, Pex14p and Pex17p, are indicated. Apart from the interaction studies hardly anything is known of the function of these candidate proteins. Remarkably, however, they should accommodate passage of folded protein domains. Several lines of evidence strongly suggest that folding of proteins is largely completed in the cytoplasm and that complete unfolding is not required for entry into the peroxisomal matrix (reviewed in McNew and Goodman, 1996). This is in line with the absence of evidence for protein folding components in peroxisomes thus far.

5. FORMATION OF PEROXISOMAL MEMBRANES

A still unanswered question is how growing and dividing peroxisomes recruit the lipids to increase the surface of the peroxisomal membranes. Three different options come to mind: i) close association with the ER, the major site of phospholipid biosynthesis in the cell, allowing transfer of lipids from ER to peroxisomes via such contact sites, ii) the use of phospholipid transfer proteins and iii) vesicular transport. Evidence for contact sites between ER and peroxisomes was found by electronmicroscopy (Novikoff and Shin, 1964) but the functional significance could not be substantiated by biochemical experiments (but see section THE ER TO PEROXISOME CONNECTION). Note, however, that the existence of contact sites between ER and mitochondria was suggested recently based on both biochemical and morphological data (Rizzuto *et al.*, 1998; Vance, 1990). Lipid transfer proteins are soluble proteins capable of transferring labeled phospholipids one at a time from a donor membrane to an unlabeled acceptor membrane in a time dependent manner. Two of these activities show phospholipid specificity against phosphatidylcholine (PC-TP) and phosphatidylinositol (PI-TP), respectively, while a third one transfers all common diacyl phospholipids, glycolipids and cholesterol (nsL-TP)(Wirtz, 1991). Based on their *in vitro* characterisation a role in transfer of phospholipids between membranes was proposed but direct evidence is lacking. Recent observations actually argue against such a role: i) these proteins mediate no net transfer from donor to acceptor membrane, ii) it is not clear how a single protein can display organelle membrane specificity considering the number of proteins needed to guide vesicles to their specific targets, iii) since they transfer a single lipid molecule at a time it is questionable whether they can mediate the large amount of lipids needed to support the biogenesis of organelles (Futerman, 1998), iv) the phosphatidyl inositol

specific transporter turned out to be encoded in yeast by *SEC14*. *SEC14* codes for a protein needed for export of secretory proteins from the Golgi (Bankaitis *et al.*, 1990). It is not immediately obvious how the *in vitro* derived properties of Sec14p can be related to the *in vivo* observed phenotype in the *sed14* mutant, finally, v) nsL-TP possesses a typical PTS1 motif at its C-terminal end and is located in peroxisomes (Keller *et al.*, 1989). A longer form, SCPx, containing nsL-TP in its C-terminus is closely associated in the matrix with acyl-CoA oxidase (Wouters *et al.*, 1998) and important for degradation of very long-chain fatty acids, particularly of branched-chain fatty acyl substrates (Seedorf *et al.*, 1998; Wanders *et al.*, 1997). SCPx/nsL-TP might help to present the hydrophobic substrates in a way that is optimal for subsequent β -oxidation. A convincing ortholog of this mammalian protein was not found in *S. cerevisiae*, which suggests that it is needed to cope with the greater variety of substrates that is encountered by mammalian peroxisomes. One would expect it to be conserved if it was involved in such a basic process as lipid recruitment by organelles. For the alternative, vesicle transfer, no evidence exists either. Renewed interest in this possibility recently arose, however, when suggestive evidence was put forward that peroxisomes or peroxisomal pre-structures might be derived from the ER.

6. THE ER TO PEROXISOME CONNECTION

The observations made in the mid-eighties that peroxisomal matrix and membrane proteins are synthesized on free polysomes and post-translationally imported into peroxisomes has led to the view that peroxisomes are autonomous organelles: they multiply by growth and division of pre-existing peroxisomes and recruit their proteins directly from the cytosol (Lazarow and Fujiki, 1985). However, recent observations challenge this model and invoke the involvement of the endoplasmic reticulum in peroxisome biogenesis. One of the first pieces of evidence referred to nowadays came from a study on the site of synthesis of peroxisomal integral membrane proteins in rat liver. Bodnar *et al.* (1991) showed that a 50 kDa peroxisomal integral membrane protein is synthesized preferentially on ER bound ribosomes. More recently, studies in yeast suggest that some peroxisomal integral membrane proteins can be found in the ER under certain conditions. Overexpression of Pex15p, a tail-anchored type II integral membrane protein, causes a profound proliferation of membranes that contain Pex15p (Elgersma *et al.*, 1997). The continuity of the proliferated membranes with the nuclear envelope suggests that they most likely originated from the ER. Final proof for the ER origin of these membranes was

obtained by double labeling experiments. Antibodies against Pex15p and an ER marker protein decorated the same membranes in yeast cells overproducing Pex15p (Stroobants, Van den Berg and Hettema, personal communication). The observation that overexpressed Pex15p is O-glycosylated, a sugar modification that in *S. cerevisiae* occurs exclusively in the ER or in ER-derived compartments, provided additional evidence for trafficking of this protein to the ER (Elgersma *et al.*, 1997). Similar results have been reported for the integral peroxisomal membrane protein Pex3p. In yeast and in mammalian cells overexpression of Pex3p leads to proliferation of ER derived membranes (Kammerer *et al.*, 1998; Elgersma *et al.*, 1997). Moreover, the first 16 amino acids of yeast Pex3p were sufficient to target a reporter protein to the ER (Baerends *et al.*, 1996). Thus, Pex3p is a possible candidate to take the ER dependent route to peroxisomes. Although suggestive, the above mentioned experiments do not exclude an artificial mislocalization to the ER caused by overexpression of the proteins. Whether the endogenous Pex15p and Pex3p travel through the ER remains to be shown.

The *PEX1* and *PEX6* genes encode proteins that belong to the so called triple A (AAA) family of proteins (reviewed in Patel and Latterich, 1998). All of them contain one or two ATP binding pockets and the most well-known examples of this family are the NSFs (NEM-sensitive factors). These proteins are implicated in vesicle trafficking between ER, Golgi and plasma membrane (Rothman and Wieland, 1996) and it is tempting to deduce a similar role for the peroxins Pex1p and Pex6p. Indeed, suggestive evidence for the presence of these proteins in small vesicles that differ from mature peroxisomes was reported in *P. pastoris* but it is not yet clear how these vesicles feature in a peroxisome biogenesis or maturation pathway (Faber *et al.*, 1998). In contrast, in mammalian cells a cytosolic location for Pex6p was found (Yahraus *et al.*, 1996).

Additional evidence implicating the ER in peroxisome assembly came from studies in the yeast *Y. lipolytica*. Titorenko *et al.* (1997) identified and characterized mutants that affect both protein secretion and peroxisome biogenesis. Some of these mutants were originally isolated by the group of Ogrydziak (Ogrydziak *et al.*, 1982). Although the nomenclature is similar to the secretion mutants collected in *S. cerevisiae* by the Schekman group, they do not necessarily have the same properties since *Y. lipolytica* differs from *S. cerevisiae* in a number of aspects. For instance, three different secretion routes have been postulated to exist based on the analysis of the various secretion mutants (Titorenko *et al.*, 1997). The *sec238* mutant is disturbed in the secretion of an extra cellular protease while the *srp54* causes abnormal function of the signal recognition particle (Lee and Ogrydziak, 1997; Ogrydziak *et al.*, 1982).

Mutations in the *SEC238*, *SRP54*, *PEX1* and *PEX6* genes not only cause defects in the exit of secretory proteins from the ER but also compromise the biogenesis of peroxisomes. More interestingly, mutations in these four genes significantly delay or prevent the exit of two peroxisomal membrane proteins, Pex2p and Pex16p, from the ER. Peroxisomal matrix proteins did not associate with the ER under these conditions. Although these results suggest that the ER is required for the assembly of functional intact peroxisomes one could argue that the observed accumulation of Pex2p and Pex16p in the ER reflects an indirect effect of the mutations. However, the same group has shown that in wild-type yeast cells endogenously expressed Pex2p and Pex16p transiently associate with the ER en route to the peroxisome (Titorenko and Rachubinski, 1998). Pulse-chase experiments followed by subcellular fractionation and immunoprecipitation revealed that Pex2p and Pex16p are located in ER enriched fractions during the pulse, and then are chased into fractions enriched in peroxisomes. In these experiments Pex2p and Pex16p were never found in Golgi enriched fractions suggesting direct ER-to-peroxisome transport of these proteins.

Further evidence for trafficking of Pex2p and Pex16p via the ER is provided by N-linked core glycosylation of both proteins. Protease protection experiments of ER enriched and peroxisome enriched fractions showed that the glycans on Pex2p and Pex16p remained intact indicating that core glycosylation of these proteins occurs in the ER lumen and that the glycosylated forms are delivered to the peroxisome. *Y. lipolytica* Pex2p contains one canonical Asn-Xaa-Thr sequence for N-linked glycosylation. This N-glycosylation site is not conserved in any of the eight Pex2p orthologues, which could provide an explanation for the fact that glycosylation of Pex2p has not been reported before. The human ortholog of *Y. lipolytica* Pex16p also lacks a consensus sequence for N-linked glycosylation (South and Gould, 1999). Furthermore, microinjection experiments in human fibroblasts failed to provide evidence for an ER role in Pex16p biogenesis: Pex16p was always detected in peroxisomes, even at the earliest time points after microinjection, and Pex16p transport to peroxisomes was not blocked or delayed in fibroblasts with an inactive *PEX1* gene or upon incubation at 15°C, a treatment that blocks exit of proteins from the intermediate compartment between the ER and Golgi (South and Gould, 1999). In addition to these differences in Pex16p biogenesis, Pex16p seems to have disparate roles in different organisms. In *Y. lipolytica* it plays a role in import of a subset of peroxisomal matrix proteins, but it is not required for membrane biogenesis (Eitzen *et al.*, 1997). In contrast, human Pex16p is required for peroxisomal membrane protein import and peroxisome synthesis (South and Gould, 1999). Caution is needed, therefore, in extrapolating the functions of a particular peroxin to its homologs in other species.

When one considers the possibility of a connection between ER and peroxisomes then vesicle transport would be a likely mechanism to establish such communication. Two observations suggest a role for ARF (ADP ribosylation factor) and coatomer in peroxisome biogenesis. Salomons *et al.* (1997) studied the effect of brefeldin A (BFA) on peroxisome biogenesis in the yeast *H. polymorpha*. BFA is a fungal toxin that prevents coatomer assembly onto membranes by inhibiting the GDP/GTP exchange activity of ARE. Immunocytochemical analysis of BFA treated cells suggested that newly synthesized peroxisomal membrane and matrix proteins accumulated at membranes that could be of ER-origin based on their continuity with the nuclear envelope. These results suggested that inhibition of recruitment of coat proteins onto membranes could interfere with peroxisome biogenesis (Salomons *et al.*, 1997). In human cells, however, peroxisome synthesis is insensitive to BFA (South and Gould, 1999).

Passreiter *et al.* (1998) found that purified rat liver peroxisomes incubated with bovine brain cytosol could recruit ARF and coatomer in a GTP- γ -S dependent manner. Further support for the involvement of coatomer in peroxisome biogenesis came from the observation that CHO cells expressing a temperature sensitive version of the e-subunit of the coatomer showed elongated tubular peroxisomes when incubated at the non-permissive temperature. The authors suggested that Pex11p, an abundant peroxisomal membrane protein that contains a cytoplasmically exposed KKKXX motif at its C-terminus, could mediate coatomer binding. It is remarkable, however, that this putative coatomer binding motif and by inference this crucial function is not conserved in the *S. cerevisiae* Pex11p ortholog. Moreover, recent observations suggest that in yeast Pex11p is involved in metabolism (see above).

7. DO PEROXISOMES POSSESS UNIQUE FEATURES?

The PTS1 and PTS2 receptors (Pex5p and Pex7p) are hydrophilic proteins and they are not associated permanently with the peroxisomal membrane. The escort model shown in Figure 1 gives for now the best explanation for their function. The concept of soluble receptors initially was received with certain reservations. Comparison with other eukaryotic protein import systems shows that this is the rule rather than the exception. The cytosolic soluble signal recognition particle picks up most of the ER targeted proteins (Walter and Johnson, 1994). Nuclear proteins are escorted to nuclear pores by the soluble receptors importin α and importin β (Görlich and Mattaj, 1996), and even for mitochondria soluble cytosolic factors for efficient protein import were found (Hachiya *et al.*, 1995). In

mitochondria they were overlooked for a long time probably because of the success of the *in vitro* import system that used purified mitochondria and because a genetic analysis of mitochondrial protein import only lately has showed more promise (Maarse *et al.*, 1992).

The concept that (partially) folded proteins can enter the peroxisome is not unique either. Folded proteins can traverse nuclear pores and pass through the plant chloroplast thylakoid membrane and bacterial inner-membrane (reviewed in Settles and Martienssen, 1998). Careful measurement of the water-filled space in the Sec61 pore of the ER indicates that folded domains in principle can be accommodated (Hamman *et al.*, 1997). The original concept that only a completely unfolded peptide chain can be threaded through a proteinaceous pore of a translocation complex is still upheld in mitochondria only (Neupert, 1997).

Finally, the observation in *Y. lipolytica* that glycosylated proteins end up in peroxisomes is not unique. Also in mitochondria a 45 kDa glycosylated protein was found (Chandra *et al.*, 1998). Pulse-chase experiments indicated that it acquired its modification in the ER before arriving in mitochondria. The paradigm is that mitochondria form by growth and division of pre-existing organelles. Because of the double membrane of the mitochondrion vesicular traffick, suggesting mitochondria to be part of the ER, is less likely to be involved. However, there may be reasons to rethink standard views on organelle biogenesis on the basis of new provocative experimental data (for a heretic opinion see Glick and Malhotra, 1998).

8. TECHNICAL SHORTCOMINGS IN THE PEROXISOME FIELD

A major handicap of peroxisomes is that they loose some of their properties after isolation. Particularly latency, a characteristic feature they display *in vivo*, is absent. The unknown causes of this damage prevented development of a reliable *in vitro* protein import system. In addition, the absence of efficient post-translational modifications of proteins upon import, such as cleavage of targeting signals, makes it even more difficult to design good import assays. As a consequence, a number of neat tricks as developed for other protein import systems cannot be applied. Attempts to translate such manipulations to the *in vivo* situation were not successful thus far. For instance, no one succeeded in designing protein constructs that would jam the peroxisomal import machinery which would allow identification of components of a protein translocation pore via chemical cross-linking with the jammed construct (Häusler *et al.*, 1996, and our own unpublished results). Digitonin or SLO permeabilized cells have been used as a compromise since (Wendland and Subramani, 1993; Rapp *et al.*, 1993).

Recently, a breakthrough was reported by the group of A. Baker using plant glyoxysomes (Pool *et al.*, 1998). The protein substrate was imported in a time-dependent and ATP-dependent manner in freshly isolated organelles as judged from acquisition of resistance to protease. Interestingly, a protein A fusion construct got stuck during membrane translocation. This would be a great tool for further studies to unravel the properties and features of protein import into peroxisomes.

An important aspect in applying the yeast "two-hybrid" protein interaction technique is that validation is obtained for a direct interaction between 'bait' and 'prey'. When the technique was used in the homologous context with yeast peroxisomal proteins it became clear that this was not an academic issue. For instance, the yeast PTS1 and PTS2 receptors (Pex5p and Pex7p) were originally suggested to interact with each other (Rehling *et al.*, 1996). This seemed reasonable since proteins with TPR repeats (Pex5p) and WD-40 repeats (Pex7p) were reported to interact with each other using genetically based experiments. Later, however, the interaction between Pex5p and Pex7p was demonstrated to be indirect and to be mediated by Pex14p, a protein associated with the peroxisomal membrane (R. Erdmann, personal communication). By carrying out the two-hybrid interactions in yeast strains with specific deletions in genes coding for peroxisomal proteins, this initial setback was turned into an advantage. For instance, in a *pex14Δ* strain the interaction between Pex5p and Pex7p is lost. By performing the interaction studies in various combinations in different genetic backgrounds the "two-hybrid" technique can be modified to act as a "three-hybrid" reporter. It is remarkable that even integral membrane proteins can act as "go betweens" in such "three-hybrid" assays.

An obvious approach to follow in studying a possible relationship between ER and peroxisomes is the application of a thermosensitive set of mutants (*sec* mutants) isolated by the Schekman group in *S. cerevisiae*. In practice this turned out to be rather difficult and meaningful results were not yet obtained: i) The parent strain used for making the *sec* mutants cannot grow on fatty acids as their sole carbon source. This is often the case with laboratory strains and is caused by mutations in genes coding for peroxisomal proteins. Probably such strains accumulated cryptic mutations in genes that are never challenged for function under growth conditions practised in most laboratories. ii) At the non-permissive temperatures required to call up the phenotype, *S. cerevisiae* anyhow grows miserably in the detergent-like fatty acid containing growth medium. iii) It is difficult to separate possible peroxisome-related events from the already traumatic events caused by a block in such an essential pathway as secretion.

A difficulty in comparing literature data is that differences in opinion are encountered whether proteins are associated with the peroxisomal

membrane or whether they are integral membrane proteins. Although all groups use the same technique, extraction of membranes with Na_2CO_3 at alkaline pH, the outcome often is different. We have observed cases in which mitochondrial membrane proteins were not extractable at all and were completely confined to the pellet fraction, whereas peroxisomal membrane proteins such as Pex15p and the ABC transporter Pat1p were 50% extractable and only 50% remained in the pellet fraction. (Elgersma *et al.*, 1997; Hettema, personal communication). The reason for such aberrant behaviour of peroxisomal membrane proteins is not clear but it does give rise to confusion in the literature.

9. OUTLOOK

The field of peroxisome biogenesis is rapidly expanding and the application of yeast genetics has unearthed many new genes coding for proteins involved in this process. Although this provides us with essential tools allowing us to catch up with the more advanced state of the art already attained with other organelles such as mitochondria, nucleus and ER, very basic questions still remain to be answered. For instance, the simple concept that peroxisomes multiply by growth and division of pre-existent ones is debated again.

How proteins are imported into peroxisomes is still a complete mystery and the first intrinsic component of the peroxisomal protein import machinery still needs to be identified. Here the advantage of an *in vitro* protein import system is desperately needed to make progress.

These critical remarks illustrate that we still have a number of challenges ahead. However, the development and application of yeast genetics to unravel aspects of peroxisome function and biogenesis has stimulated the field considerably and has already paid off for further understanding of the pathology of peroxisomes in man.

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Chapter 9

Neurons, Chromaffin Cells and Membrane Fusion

Peter Partoens, Dirk Slembrouck, Hilde De Busser,
Peter F. T. Vaughan, Guido A. F. Van Dessel,
Werner P. De Potter, and Albert R. Lagrou

1. INTRODUCTION

It is now established that each neuron can secrete a variety of peptidergic and non-peptidergic/classical transmitters via at least two types of secretory organelles, i.e. the small synaptic vesicles (SSVs; 40–60nm diameter) and the large dense-cored vesicles (LDVs; >75nm diameter), also referred to as secretory granules. According to the current model, the

HILDE DE BUSSER and ALBERT R. LAGROU RUCA-Laboratory for Human Biochemistry, Department of Biochemistry, Physiology and Genetics, UA-Faculty of Medical and Pharmaceutical Sciences, University of Antwerp, B-2020 Antwerp, Belgium. **GUIDO A. E VAN DESSEL** UIA Laboratory for Pathological Biochemistry, Department of Medicine, UA-Faculty of Medicine and Pharmaceutical Sciences, University of Antwerp, B-2610 Wilrijk-Antwerp, Belgium. **PETER F. T. VAUGHAN** Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, United Kingdom. **PETER PARTOENS, DIRK SLEMBROUCK, and WERNER P. DE POTTER** UIA Laboratory for Neuropharmacology, Department of Medicine, UA-Faculty of Medicine and Pharmaceutical Sciences, University of Antwerp, B-2610 Wilrijk-Antwerp, Belgium.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

classical neurotransmitters such as ACh, glutamate and GABA are stored in, and released from, the SSVs (for review see De Camilli and Jahn, 1990). These vesicles originate from the endosomal compartment (Régnier-Vigouroux and Huttner, 1993) and are continuously regenerated by local recycling. The neuropeptides, on the other hand, are stored in, and released from, the LDVs (Winkler and Fischer-Colbrie, 1990). Intensive work on SSVs has led to a good insight in their biogenesis and a better understanding of the molecular mechanisms underlying vesicle recycling, such that a first glimpse of the exocytosis/endocytosis cycle is starting to emerge. For an extensive view on characteristics of SSVs as well as the SSV-cycle, the reader is referred to other excellent reviews in this volume. In this review we want to focus on the biochemical properties and the intraneuronal dynamics of LDVs of noradrenergic neurons and the—quite similar—secretory granules of the chromaffin cells.

In 1953 it was demonstrated by Blascko and Welch that in the adrenal medulla catecholamines were stored in subcellular organelles called chromaffin granules. Further research revealed that these granules contain specific soluble proteins, the chromogranins (see Kirschner and Kirschner, 1971) and membrane proteins (see Winkler, 1971). Biochemical and immunochemical studies directly led to the finding that, upon stimulation of the adrenal medulla these chromogranins are released by the mechanism of exocytosis (see Kirschner and Kirschner, 1971).

Although it was already proposed by Scott in 1905 that “nerves act upon one another and other cells by the passage of a chemical substance of the nature of a ferment or proferment” in other words by secretion of an enzyme or a protein, it was only in 1969 that, in analogy with the chromaffin cells, also (splenic) nerves were actually shown to secrete proteins. The proteins, chromogranin A and the soluble form of the enzyme dopamine β -hydroxylase (DbH), present in the LDVs, were shown to be released upon stimulation from sympathetic neurons together with noradrenaline (NA), while the enzymes tyrosine hydroxylase and lactate-dehydrogenase, present in the cytoplasm, were not released (De Potter *et al.*, 1969; Smith *et al.*, 1970). Despite these important findings, LDVs were considered for many years to be of little physiological importance and not eminently involved in chemical neurotransmission (Basbaum and Heuser, 1979; Pollard *et al.*, 1982). It was only after the demonstration of the presence of peptides as NPY and enkephalins in LDVs and consequently their co-release with NA, that the interest in LDVs was renewed (Lundberg and Hockfelt, 1983; Lundberg *et al.*, 1989). Further studies led to the concept of differential release from which it was inferred that LDVs release their

content only at higher frequencies of stimulation (Bartfai *et al.*, 1988). Although this concept has now been generalized, it has, at least for noradrenergic neurons, been challenged recently. By using different approaches, it is demonstrated that the release of the neurotransmitters from sympathetic neurons ultimately occurs from the LDVs (De Potter *et al.*, 1995; 1997).

Since the original demonstration of the co-storage of chromogranins and NA in secretory granules in chromaffin cells, many studies revealed that most hormones and also neurotransmitters are stored in specialized organelles, called endocrine secretory granules in various endocrine tissues and LDVs in the nervous tissue. For a long time it was assumed that all these vesicles, storing various hormones and transmitters, were different from each other in their biochemical composition but evidence accumulates that the opposite is true. Various proteins (chromogranins) and neuropeptides originally confined to chromaffin granules and LDVs are found in endocrine secretory granules. It is now generally established that LDVs, chromaffin granules and endocrine secretory granules, apart from their respective hormones and transmitters, exhibit similar properties (for review see Winkler and Fischer-Colbrie, 1990). In this review we will discuss the properties of their membrane proteins, with respect to their role in exocytosis, based on studies on chromaffin cells, the human neuroblastoma cell SH-SY5Y and primary cultures of noradrenergic neurons.

Because of their common ontology with peripheral noradrenergic neurons and their large yield, primary cultures from bovine chromaffin cells have been used extensively as a model to study the physiological and morphological aspects of sympathetic adrenergic neurons.

The neuroblastoma clone SH-SY5Y derived from a human sympathetic ganglion (Ross and Biedler, 1985) is becoming of increasing importance as a model for a sympathetic neuron. Thus previous studies have shown that SH-SY5Y can be differentiated to express many properties of a mature sympathetic neuron (Pahlman *et al.*, 1990). For example treatment of SH-SY5Y cells for several days with low concentrations (16 nM) of TPA, resulted in an increased synthesis of NA leading to a higher content of NA compared to DA. In addition, preliminary studies demonstrated that TPA treated SH-SY5Y cells expressed high affinity uptake and depolarisation-evoked Ca^{2+} -dependent release of NA.

A primary culture of peripheral noradrenergic neurons, obtained from the porcine ganglion superior cervicalis (GSC), was introduced a few years ago (Wang *et al.*, 1995; Wang and De Potter, 1998). The larger yield of this culture provides the possibility to study noradrenergic neurons not only microscopically but also biochemically.

2. BIOGENESIS AND AXONAL TRANSPORT OF LDV/SECRETORY GRANULES

LDVs and secretory granules are formed at the trans-Golgi network (TGN) in the cell body, in contrast to SSVs which originate locally at the synapse. At the TGN exit site the selective sorting of potential vesicle matrix proteins to the immature secretory vesicles of the regulated secretory pathway takes place. It is generally accepted that co-aggregation of abundant membrane proteins with matrix proteins is the mechanism behind this sorting and retention of secretory vesicle proteins during vesicle formation and maturation (Tooze *et al.*, 1993).

The chromogranins A and B (CGA; CGB), the major proteins of the secretory granules have been shown to aggregate in a low pH and a high calcium environment, the conditions found in the trans-Golgi network. Moreover, CGA and CGB, as well as several other secretory vesicle matrix proteins, have been shown to bind to the vesicle membrane at the intravesicular pH of 5.5 and to dissociate from it at a near physiological pH of 7.5. Furthermore, most of the vesicle matrix proteins bind not only to the vesicle membrane but also to CGA at pH 5.5, with the exception of a few matrix proteins that appear to bind only to CGA or to the vesicle membrane (Yoo, 1994,1995; 1996).

It is not completely clear how vesicle matrix proteins could interact with the granule membrane proteins. The existence of a receptor that on one hand recognizes the aggregated vesicle matrix proteins and on the other hand interacts with the granule membrane proteins has been postulated (Bauerfeind *et al.*, 1994). For CGB, a receptor recognition site, known as the S-M (secretory membrane recognition) signal, has been suggested (Chanat *et al.*, 1993). However, sequence comparison of a wide range of vesicle matrix proteins has not revealed a consensus sequence that qualifies as an S-M signal (Bauerfeind *et al.*, 1994).

After their formation at the TGN in the neuronal cell body, immature secretory granules have to be transported to their release sites at the nerve endings through the nerve axon. This, for the survival and function of the neuron crucial, intra-axonal transport happens by fast axonal transport. Fast axonal transport of membranous organelles has clearly been demonstrated to be a microtubule-dependent process (Allen *et al.*, 1985; Vale *et al.*, 1985). Most axonal microtubules are arranged with their plus ends towards the terminals (Heidemann *et al.*, 1981), thus providing a substrate for organelle movement. Colchicine, which binds to tubuline, inhibits normal vesicle transport (Dahlstrom 1968; 1970). Kinesine is the motor-protein for anterograde transport (Vale *et al.*, 1985). Antibodies to kinesin inhibit axonal transport in the squid

axoplasm (Brady *et al.*, 1990). Evidence has shown that ATP is involved in fast axonal transport and ATP hydrolysis supplies the power for axonal transport (Ochs, 1971).

3. EXOCYTOSIS FROM LDV/SECRETORY GRANULES

In non-noradrenergic neurons LDVs are rare and the major organelle is represented by the small synaptic vesicle. SSVs are very abundant in the central nervous system where they can be prepared with a high degree of purity. This led in recent years to a thorough characterization of this vesicle. In particular, the identification and characterization of the membrane proteins present on SSVs (and the plasmalemma) has led to a general model of membrane fusion and has revealed the molecular mechanisms involved in vesicle recycling. We do not have the intention to give a complete overview on the molecular mechanisms governing the exocytotic process. The reader is therefore referred to other chapters in this book. We will limit ourself to a description of the LDV/secretory granule membrane composition from which it can be concluded that the crucial actors governing SSV-exocytosis are also responsible for LDV exocytosis. We will emphasise the role of the cytoskeleton in the exocytosis of LDVs, the role of GTP-binding proteins in LDV-exocytosis and the related isoprenylating/ carboxymethylating mechanisms.

3.1. The Membrane Composition of Secretory Vesicles/LDV

Knowledge about the biochemical properties of LDVs comes mainly from analysis of LDV-enriched preparations obtained from the bovine splenic nerve and from the closely related secretory granules of the chromaffin cells. An overview, based on a search through literature, of the intrinsic and cytosolic membrane-associated proteins present on secretory granules/LDV is given in Table 1. We limit ourselves to those proteins which have been postulated to play a role in regulated exocytosis, either by functional studies or by their characteristics.

Membrane proteins of LDVs of particular interest in this review are (i.) those thought to be directly involved in the mechanism of exocytosis and/or (ii.) those which can be used as markers to follow the exo-endocytosis coupling as such. These proteins will be discussed in detail below.

To the first category belong a number of proteins present on SSV and shown to play a role in the vesicular fusion machinery, such as synaptotagmin, synaptobrevin and synaptophysin.

Table 1
Overview of the integral membrane and cytosolic membrane-bound
proteins present on secretory granules/LDV

Membrane proteins	chromaffin granules	LDV	Reference
annexins Ca ²⁺ - and phospholipid-binding multifunctional proteins implicated in membrane transport	+		Ali, S. M. <i>et al.</i> , 1989; Drust, D. S. and Creutz, C. E. J., 1991; Creutz, C. E. <i>et al.</i> , 1992; Bandorowicz- Pikula, J. and Pikula, S., 1998
annexin II / calpactin several characteristics suggest its involvement in regulated exocytosis although its function in exocytosis has been refuted by others; annexin II has also been implicated in NEM- independent vesicular fusion processes in epithelial cells			
annexin VI / p68 work on annexin VI has mainly addressed its possible role in the endocytic pathway			
annexin VII / synexin <i>in vitro</i> annexin VII aggregates chromaffin granules and enhances membrane fusion in a Ca ²⁺ - and GTP- dependent manner			
aminophospholipid translocase subfamily of P-type ATPases; provides ATP-dependent transport of phospholipids; the mechanism to maintain phosphatidylserin orientation was suggested to be important for Ca ²⁺ - dependent binding of annexin (Zachowski, A. <i>et al.</i> , 1989)	+		Devaux, P. F. <i>et al.</i> , 1990
CAPS (Ca ²⁺ -dependent activator protein of secretion) identified as a required factor for brain LDV and secretory granule secretion; it is absent from SSV and not involved in SSV exocytosis; it shows a specific binding with acidic phospholipids	+	+	Walent <i>et al.</i> , 1992; Ann, K. <i>et al.</i> , 1997; Benvin, B. <i>et al.</i> , 1998

phosphatidylinositol 4-kinase (PI-4 kinase)	+		Husebye, E. S. <i>et al.</i> , 1990
its activity is required for stimulated secretion (Wiedemann, C. <i>et al.</i> , 1996); PI-4 kinase enables the site-specific synthesis of PIP2 which has several candidate binding proteins, including CAPS and synaptotagmin (Martin, T. E. J. 1997)			
synaptotagmin (p65)	+		Lowe, A. W. <i>et al.</i> , 1988;
regulates stimulus-secretion coupling in regulated exocytosis; it is the most likely candidate as Ca ²⁺ -sensor in regulated exocytosis	+	+	Fournier, S. <i>et al.</i> , 1989;
		+	Schmidle, T. <i>et al.</i> , 1991;
		+	Annaert <i>et al.</i> , 1997
sv2	+		Lowe, A. W. <i>et al.</i> , 1988;
a proteoglycan found in SSV and LDV; its amino acid sequence suggests it is a vesicular transporter; a calcium-regulated interaction with synaptotagmin is also reported (Schivell, A. E. <i>et al.</i> , 1996)		+	Schmidle, T. <i>et al.</i> , 1991
synaptophysin (p38)	+/-	+/-	see Winkler <i>et al.</i> , 1997
transmembrane glycoprotein; suggested to interact with the fusion complex in SSV exocytosis			
VAMP / synaptobrevin (p18)	+		Hohne-Zehl, B. <i>et al.</i> , 1994;
vesicular SNAP receptor; crucial for formation of the fusion complex			Hodel <i>et al.</i> , 1994;
			Foran <i>et al.</i> , 1995
cellubrevin	+		Foran, P. <i>et al.</i> , 1995
synaptobrevin homologue; vesicular SNAP receptor for intracellular fusion events; colocalized with synaptobrevin			
N-ethylmaleimide sensitive fusion protein (NSF)	+		Burgoyne, R. D. en
soluble NSF attachment protein (SNAPs)	+		Williams, G., 1997
NSF en SNAPs associate with their membrane receptors only when vesicles dock on the target membrane			
SNAP-25			Hohne-Zehl, B. and
• SNAP-25 monomers	+		Gratzl, M., 1996
• SNAP-25/syntaxin heterodimers	+		
target SNAP receptor on plasma membrane; SNAP-25 on chromaffin granules has essentially the same properties as does SNAP-25 on the plasma membrane			

(continued)

Table 1
Continued

Membrane proteins	chromaffin granules	LDV	Reference
syntaxin 1 target-SNAP receptor on plasma membrane; its presence on granules suggest it could also function as a vesicular SNAP receptor	+		Tagaya, M. <i>et al.</i> , 1995
Rab3d/a-d suggested to regulate the efficiency of exocytosis	+	+	Darchen <i>et al.</i> , 1990; Annaert <i>et al.</i> , 1997
rabphilin 3a regulator of exocytosis	+		Chung <i>et al.</i> , 1995
calmodulin intracellular Ca^{2+} -receptor, membrane bound; bound with high affinity to granule membrane proteins of Mrs 25 and 22 kDa at low Ca^{2+} (less than 10-8 M) and two proteins with Mrs 69 and 50 kDa at high Ca^{2+} (greater than 1 μM); one of the calmodulin binding proteins was later identified as synaptotagmin / p65 (Fournier, S. and Tkifaro, J. M., 1988; 1989)	+		Geisow, M. J. and Burgoyne, R. D., 1983; Hikita, T. <i>et al.</i> , 1984
cytoskeleton proteins a number of cytoskeleton proteins have been shown to bind to the chromaffin granules and are proposed to have a role in secretion			
F-actin (membrane-bound)	+		Meyer, D. I. and Burger, M. M., 1979; Fowler, V. M. and Pollard, H. B., 1982
spectrin (membrane-bound)	+		Aunis, D. and Perrin, D. J., 1984
alpha-actinin	+		Jockusch, B. M. <i>et al.</i> , 1977; Trifaro <i>et al.</i> , 1984
Chromobmdin A / CCT a large, multisubunit protein that binds to chromaffin granule membranes in a Ca^{2+} - and ATP-regulated manner. Ca^{2+} stimulates binding to the membrane, whereas ATP, in the absence of Ca^{2+} , is required for release of the protein from the membrane	+		Martin, W. H. and Creutz, C. E., 1990; Martin, W. H. and Creutz, C. E., 1987; Creutz, C. E. <i>et al.</i> , 1987

chromobindin A was recently identified as the cytosolic chaperonin CCT (chaperonin containing TCP-1), which has a known function in tubulin and actin conformation (Creutz, C. E. *et al.*, 1994)

The data above suggest that the proteins demonstrated to be present on chromaffin granules will also be found on neuronal LDV. Since, as is already mentioned above, it is generally accepted that LDVs, chromaffin granules and endocrine secretory vesicles exhibit similar properties (for review see Winkler and Fischer-Colbrie, 1990), it can be predicted that the proteins summed up in table 1 will be present also on endocrine secretory granules. In this respect the presence of Rab3 on zymogen granules (Jena *et al.*, 1994) is worth mentioning. In addition, these data point to a basic mechanism of exocytosis, similar for synaptic vesicles, LDVs, chromaffin granules and endocrine secretory vesicles.

—Synaptotagmin, also called p65, is a transmembrane glycoprotein, which cytoplasmic tail (C-terminus) contains two copies of a sequence displaying homology to the Ca^{++} -binding domain of protein kinase C. Since the Ca^{++} -sensor synaptotagmin interacts with adaptor protein II (AP2), neurexins and syntaxins, a regulatory role in diverse synaptic events including docking and fusion of SSVs and endocytosis of retrieved SSV-membranes is suggested. It is demonstrated to be present also in chromaffin granule (Schmidle *et al.*, 1991; Trifaro *et al.*, 1989) and LDV membranes (Schmidle *et al.*, 1991).

—Synaptobrevin, also called p18 or vesicle associated-membrane protein (VAMP) is a crucial actor in the vesicular fusion machinery. It is anchored to and extends from the cytoplasmic surface of the SSV. It is selectively cleaved by tetanus toxin and botulinum toxin B, D, F and G, both Zn^{++} -metalloproteases and potent inhibitors of exocytosis. Synaptobrevin forms a fusion complex with the presynaptic membrane proteins SNAP-25 and syntaxin. Since tetanus toxin affects not only the release from SSV but also from secretory granules in the adrenal medulla (Höhne-Zell *et al.*, 1994; Foran *et al.*, 1995), it could be predicted that synaptobrevin is also present on secretory granules. Indeed, Goodall *et al.* (1997) demonstrated the presence of synaptobrevin on LDVs in SH-SY5Y cells. Although, originally it was believed that LDVs contain only very small amounts of this protein (De Camilli and Jahn, 1990), it was later demonstrated that secretory granules of the chromaffin cell contain significant amounts of synaptobrevin. In fact, it was demonstrated that the membranes of the chromaffin granules have about one third of the synaptobrevin content of the synaptic vesicles (Foran *et al.*, 1995).

—Synaptophysin, also called p38, is the most abundant integral membrane protein of SSVs in almost all nerve terminals. This protein has four

transmembrane domains with both the N- and C-terminus extending into the cytoplasm. Synaptophysin has been reported to bind to synaptobrevin. Its precise role is still not clear. A role as component of the fusion pore is proposed but also involvement in the biogenesis, recycling and stability of SSVs is possible.

A large number of conflicting reports exist concerning the presence of synaptophysin on LDVs (for a review of the discussion, see Winkler, 1997). It seems however that if synaptophysin is present on LDVs, its concentration will be low.

Membrane-bound LDV-proteins that can serve as markers for the LDV-membranes in the exo-endocytotic pathway are

- i.) The components of the monoamine uptake machinery; the vesicular monoamine transporter 2 (VMAT2) (Erickson and Eiden, 1993) and a proton translocating vacuolar ATPase (V_0 -ATPase) (Schmidt *et al.*, 1982), generating the electrochemical gradient for driving the uptake of monoamines and nucleotides.
- ii.) The components of the NA biosynthesis machinery; DbH (Smith *et al.*, 1970; Hörtnagl *et al.*, 1972) and cytochrome b_{561} (Flatmark *et al.*, 1971; Schwarzenbrunner *et al.*, 1990).

3.2. The Role of the Cytoskeleton in Secretion from LDV

3.2.1. The Human Neuroblastoma SH-SY5Y as a Model to Study the Role of the Cytoskeleton in Secretion from LDV

In this chapter the effect of activation of PKC on NA release from the human neuroblastoma SH-SY5Y will be considered in relation to the hypothesis that a key step is the rearrangement of the F-actin cytoskeleton. The neuroblastoma clone SH-SY5Y, derived from a human sympathetic ganglion (Ross and Biedler, 1985), is becoming of increasing importance as a model for a sympathetic neuron. Thus previous studies have shown that SH-SY5Y can be differentiated to express many properties of a mature sympathetic neuron (Pahlman *et al.*, 1990). For example treatment of SH-SY5Y cells for several days with low concentrations (16nM) of TPA, resulted in an increased synthesis of NA leading to a higher content of NA compared to DA. In addition preliminary studies found that TPA treated SH-SY5Y cells expressed high affinity uptake and depolarisation-evoked Ca^{2+} dependent release of NA (Scott *et al.*, 1986). Furthermore several groups have established the value of SH-SY5Y as a model for studying the regulation of differentiation by PKC (Heikkilä *et al.*, 1989; Leli *et al.*, 1992;

Parrow *et al.*, 1995) leading to the observation that down regulation of PKC- α and - ϵ was incompatible with differentiation. In addition recent studies have suggested that SH-SY5Y is a convenient cell line for determining the molecular mechanisms underlying the regulation of NA release by PKC. Thus SH-SY5Y expresses high affinity uptake of NA and glutamate (O'Neil *et al.*, 1994) and depolarisation (induced by raised K^+ , veratridine, nicotinic agonists and Ba^{2+}) and Ca^{2+} ionophore (A23187) evoked release of [3H]NA which is dependent on extracellular Ca^{2+} . Furthermore activation of muscarinic (M_3), bradykinin (B_2) and (in cells transfected with the rat AT1A receptor) angiotensin II receptors also evoke NA release by a mechanism that largely uses intracellular Ca^{2+} (Murphy *et al.*, 1991; Vaughan *et al.*, 1993; 1995; McDonald *et al.*, 1994; McDonald *et al.*, 1995a). An important difference between these studies and previous work is that NA release is measured in cultures of SH-SY5Y cells which have not been differentiated by exposure to phorbol esters for several days (Vaughan *et al.*, 1995). Several groups have found that SH-SY5Y cells express both L- and N-type Ca^{2+} channels and that NA release is inhibited by L-type (nifedipine) and N-type (w-conotoxin) VSCC antagonists which suggests that both channel types couple to NA release (Vaughan *et al.*, 1997).

SH-SY5Y cells express muscarinic M_1, M_2 (Adem *et al.*, 1987) and M_3 (Lambert *et al.*, 1989) receptor subtypes as well as μ - and δ - opioid receptors (Kazmi and Mishra, 1987) and α_2 -adreno receptors (Kazmi and Mishra 1989). More recently several groups (McDonald *et al.*, 1994; Vaughan *et al.*, 1995; Purkiss *et al.*, 1995) have provided electrophysiological evidence and NA release data which suggest that SH-SY5Y expresses bradykinin B_2 (McDonald *et al.*, 1994; Purkiss *et al.*, 1995), muscarinic M_1 (McDonald *et al.*, 1994) and neuropeptide Y (NPY) Y_2 receptors (McDonald *et al.*, 1995 a,b). SH-SY5Y also expresses the G proteins $G_{s\alpha}$, $G_{i\alpha 1}$, $G_{i\alpha 2}$, $G_{o\alpha}$ $G_{z\alpha}$ and G_b . $G_{o\alpha}$ is the major G protein found in the CNS and $G_{z\alpha}$ appears to be closely associated with neuronal tissue (Ammer and Schultz 1994). Thus SH-SY5Y cells express several G proteins found in the mammalian nervous system (Mereray and Bennett, 1995). In addition the observations that activation of muscarinic M_3 and bradykinin B_2 -receptors is accompanied by changes in inositol phosphates (Purkiss *et al.*, 1995; Lambert and Nahorski, 1990) and [Ca^{2+}] $_i$ (McDonald *et al.*, 1994; Purkiss *et al.*, 1995; Lambert and Nahorski, 1990; Murphy *et al.*, 1995) suggest that G proteins are important in the regulation of [Ca^{2+}] $_i$ in SH-SY5Y. Additional support for this view is provided by the observations that muscarinic (M_3) and NPY (Y_2) inhibition of Ca^{2+} currents is inhibited by pertussis toxin (McDonald *et al.*, 1995b,c; Reeve *et al.*, 1995).

Release of NA is maximally enhanced by 8 min pretreatment with TPA (100nM) which activates PKC (Murphy *et al.*, 1992). Recent studies (Turner

et al., 1994; 1996) using western blots have detected PKC subtypes α , ϵ and ζ in SH-SY5Y. Furthermore pretreatment of SH-SY5Y cell layers with phorbol 12,13-dibutyrate for 48 hr results in the differential down regulation of PKC- α by over 90% compared with PKC- ϵ (approximately 50%) and little effect on expression of PKC- ζ as detected by western blots (Turner *et al.*, 1996). These differences have been refined by comparing the effect of PDBu and bryostatin-1 (previously found to down regulate PKC in SH-SY5Y Jalava *et al.*, 1993) on the expression of PKC subtypes. Treatment with PDBu and bryostatin has proved particularly useful as PKC- α is decreased by 40 and 60% respectively after 2 hr whereas PKC- ϵ and - ζ are not affected. Since acute exposure to TPA no longer enhances NA release in cells exposed to bryostatin-1 for 2 hrs (Turner *et al.*, 1996) it is probable that PKC- α is the important subtype mediating the enhancement of NA release in SH-SY5Y cells. These findings have led to the establishment of conditions under which activation of PKC will enhance release and thus to identify PKC substrates important in this process in terms of a) their phosphorylation time, b) their intracellular relocation and c) how these events are affected by selective down regulation of PKC- α (Goodall *et al.*, 1997a). A more detailed account of these changes will be presented later. The observation that TPA enhances NA release in SH-SY5Y is in agreement with other workers using synaptosomes, brain slices and primary cultures of striatal neurones (Robinson, 1991) who have also reported enhancement of neurotransmitter release following activation of PKC by phorbol esters. The human neuroblastoma SH-SY5Y thus provides us with an ideal cell line with which to study the role of PKC substrates associated with the cytoskeleton, vesicles and plasma membranes in the regulation of NA release.

3.2.2. Cytoskeletal and Vesicular Proteins and Exocytosis

Current hypotheses (Jahn and Südhof, 1994; Südhof and Jahn, 1991; Trifaro and Vitale, 1993) propose that SSVs accumulate in distinct regions of the cytoplasm (active zones) and that only those vesicles apposed to the inner layer of the plasma membrane are able to release their contents in response to the rise in $[Ca^{2+}]_i$. The bulk of the vesicles are prevented from diffusion to the plasma membrane by interaction with cytoskeletal proteins such as F-actin. For example, synapsin I is a neuron-specific vesicle associated protein that is phosphorylated in response to depolarisation. In the dephosphorylated state synapsin I binds SSVs to F-actin and thus traps the vesicles in a cytoskeletal mesh. One consequence of depolarisation is activation of the Ca^{2+} /calmodulin dependent protein kinase (CAM kinase II)

resulting in the phosphorylation of synapsin I. This leads to the liberation of vesicles from their links with F-actin and increased migration of vesicles to the plasma membrane (De Camilli *et al.*, 1990; Jahn and Südhof, 1994; Trifaró and Vitale, 1993).

A different scenario occurs for LDVs including chromaffin granules which are regarded as a convenient model for studying exocytosis from LDVs. Current hypotheses (Cheek and Burgoyne, 1986; Trifaró and Vitale, 1993; Vitale *et al.*, 1995) suggest that under resting conditions the majority of secretory granules are located at least 150nm from the plasma membrane in chromaffin cells. This suggests that there is a barrier preventing the movement of vesicles to the plasma membrane. Immunocytochemical studies show that chromaffin cells have a continuous ring of F-actin underneath the plasma membrane. Furthermore, quantitative electron microscopic studies show that in any given section of a resting chromaffin cell less than 10% of granules are seen within 50nm of the plasma membrane. Following treatment with 100nM TPA this number increases to 12–15% (Vitale *et al.*, 1995). Chromaffin granules are retained within the cortical actin network where they are associated with F-actin by α -actinin (Trifaró *et al.*, 1992). The F-actin filaments are linked to the plasma membrane by fodrin (also known as β II-spectrin a member of the spectrin family of proteins which links F-actin to plasma membranes in most cells). Additional support for the general hypothesis is provided by the recent studies of Gillis *et al.* (1996) who used membrane capacitance measurements to assay Ca^{2+} -triggered exocytosis in single bovine adrenal chromaffin cells. Treatment with TPA increased the size of the readily releasable pool of secretory granules. Thus TPA increased the amplitude but not the time course of the exocytotic burst that resulted from rapid elevation of $[\text{Ca}^{2+}]_i$ due to photolysis of DMI-nitrophen (“caged” Ca^{2+}). The authors conclude that PKC affects a late step in secretion but not the Ca^{2+} sensitivity of the final step.

An alternative mechanism proposes a role for the calmodulin-binding, F-actin regulatory protein caldesmon as well as the Ca^{2+} -dependent F-actin severing enzymes scinderin and gelsolin. At low $[\text{Ca}^{2+}]_i$ caldesmon stimulates actin assembly and cross links F-actin to form an actin “cage” which provides a barrier to exocytosis (Aunis and Bader, 1988). Entry of Ca^{2+} following depolarisation activates calmodulin which binds to caldesmon leading to a disruption of the actin cross links. Thus increasing $[\text{Ca}^{2+}]_i$ not only disrupts links between vesicles and F-actin but also dissolves the cytoskeletal gel. There is good evidence showing that following K^+ -evoked or nicotinic receptor stimulation-induced depolarisation the sub-plasmalemmal F-actin disassembles allowing the secretory granules in

chromaffin cells to reach the plasma membrane (Trifarò and Vitale, 1993). Studies by Trifarò *et al.* have suggested that the major cause of F-actin disassembly following depolarisation is activation of the Ca^{2+} -dependent F-actin severing enzyme, scinderin, similar to gelsolin and villin (Trifarò *et al.*, 1992; Marcu *et al.*, 1994). In support of this view are the observations that scinderin, but not gelsolin, diffuses out of permeabilized chromaffin cells and that loss of scinderin is accompanied by a loss of chromaffin cell secretory response (Vitale *et al.*, 1992). Furthermore Vitale *et al.* (1991) reported that following depolarisation of chromaffin cells due to activation of nicotinic receptors or elevated K^+ subcortical scinderin but not gelsolin redistributes to foci on the plasma membrane and this redistribution precedes catecholamine secretion. More recently Zhang *et al.* (1996) have shown that recombinant scinderin potentiates F-actin disassembly and exocytosis in permeabilised chromaffin cells and that this action is blocked by two peptides with sequences corresponding to two F-actin binding sites on scinderin. In addition studies on tissue distribution suggest that scinderin is restricted to tissues with high secretory activity (Tchakarova *et al.*, 1990).

Treatment of chromaffin cells with phorbol esters causes a partial disruption of the cortical actin cytoskeleton, increases the number of LDVs in the 0–50 nm cortical zone and potentiates nicotine-, potassium- and electrically-evoked catecholamine release (Vitale *et al.*, 1995). These observations together with the inhibitory effect of cyclic AMP on nicotine-induced F-actin disassembly and secretion, suggests that cortical actin networks are also a site for second messenger modulation of exocytosis.

Additional support for a role for calmodulin in release from LDVs was provided by the study of Schweitzer *et al.* (1995) on NA release from PC12 cells. In this study it was reported that the Ca^{2+} /calmodulin kinase II inhibitor, KN-42, inhibited approximately 50% of NA release evoked by either carbachol or direct depolarisation. KN-42 did not inhibit the rise in $[\text{Ca}^{2+}]_i$ observed following depolarisation or application of carbachol. The authors conclude that two pools of LDVs occur in PC12 cells one of which is able to release NA in response to a rise in $[\text{Ca}^{2+}]_i$. Release from the other pool requires a Ca^{2+} /calmodulin kinase II dependent step in addition. Clearly this pool of LDVs has analogies to the requirement for phosphorylation of synapsin-I in the liberation of the reserve pool of SSVs from F-actin in central synapses.

Additional support for the hypothesis that F-actin acts as a barrier to secretion from LDVs is provided by the recent study of Chen and Wagner (1997) which found that K^+ and ATP-evoked release of NA from PC12 cells is accompanied by a Ca^{2+} -dependent decrease in F-actin.

3.2.3. Candidate Target Proteins for PKC Substrates

The actin cytoskeleton is implicated in several functions e.g. cell locomotion, cytokinesis, cell-cell and cell substratum interactions, localisation of subcellular components such as multi-protein signalling complexes and vesicular and organelle transport. It is this latter function which will be considered in this chapter. This multiplicity of function suggests that there will be distinct F-actin structures *in vivo*, each with unique and functionally significant biophysical and biochemical properties. A key question to be addressed is how the site specific assembly and disassembly of F-actin may be regulated by a) activation of kinases particularly PKC and increases in $[Ca^{2+}]_i$. In order to gain an insight into this process it is necessary to review the various mechanisms which have been put forward to account for the way cells regulate the growth of the F-actin cytoskeleton. For more details the reader is referred to articles in a recent issue of *Current Opinion in Cell biology*, 1998, volume 10, Cytoskeleton.

The formation of different actin assemblies is controlled by a large number of actin regulatory proteins, which are responsible for the cross linking, severing and capping of actin filaments and for site specific filament growth. Furthermore mechanisms for the rearrangement of the attachment of F-actin to plasma membranes (Le anchoring proteins) are likely to be more relevant than mechanisms for break down and resynthesis of filaments. In a recent review, Puius *et al.* (1998) describe the structures of the actin regulatory proteins involved in three types of regulatory mechanisms: cross linking, severing/capping-uncapping and site-specific filament assembly.

Cross Linking. Protein cross linkers e.g. fimbrin, α -actinin, dystrophin, spectrin and MARCKS are important for the ordering of F-actin into higher structures. Crosslinkers which have two F-actin binding sites in close proximity direct the formation of tight bundles, those in which the two F-actin binding sites are more distant lead to looser assemblies.

Once crosslinked F-actin complexes are formed they are likely to be too stable to be greatly affected by changes in protein concentration (as is suggested to account for treadmilling see below). In the case of fimbrin and α -actinin, F-actin binding is regulated by Ca^{2+} binding to EF domains (such that increases in Ca^{2+} decrease binding to F-actin). Recent crystallographic studies on human fimbrin suggest that an important F-actin binding region is the CH domain (CH= calponin homology after the muscle regulatory protein calponin which has been adopted as the family prototype). In fibrin the actin binding domain spans approximately 250 amino acids and consists of a tandem repeat of two CH domains which are adjacent to the EF domains. Each CH domain is composed of six helical segments and have

been shown by electron microscopic reconstructions to form the vast majority of contacts with F-actin. Interestingly it is reported that single CH regions are found in many signalling proteins. Thus it is possible that a function of single CH domains is to localise signalling molecules to the actin cytoskeleton.

Severing, Capping and Uncapping. Polymerisation or growing ends of F-actin (free barbed ends) are necessary for all actin-based motility processes as they allow the regulated assembly/ disassembly of actin filaments. Filament-severing proteins are typified by the gelsolin and cofilin families both of which bind to G- and F-actin, via a 15 kDa domain and sever actin filaments. These proteins thus play an important regulatory role in the assembly/ disassembly of actin filaments. In addition gelsolin caps actin filaments.

Ca^{2+} is required for actin severing by gelsolin (Yin and Stossel, 1979). In contrast polyphosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2) inhibit the *in vitro* severing and capping activities of gelsolin. Thus the binding to G- and F-actin to gelsolin is regulated by Ca^{2+} and phospholipids. The cofilin family of proteins (homologous with the actin-depolymerising, ADF family) bind G- and F-actin. They possess pH-dependent severing activities, enhance the rate of actin filament turnover and have little sequence homology to gelsolin. The depolymerising of F-actin by ADF/cofilin is inhibited by PIP_2 but not to Ca^{2+} .

Monomer Binding Proteins. An essential feature of cytoskeletal regulation is the site-specific assembly of actin structures. The G-actin binding protein profilin regulates the dynamics of actin polymerisation and the formation of specific F-actin structures *in vivo*. Possibly through enhancement of ADP/ATP exchange and the promotion of ATP-dependent addition of G-actin monomers to the barbed end of F-actin. In addition to binding actin profilins bind to poly-L proline in the range 10-100 μM as well as to several proline rich proteins including the focal adhesion vasodilator-stimulated phosphoprotein (VASP). This raises the possibility that proline rich proteins might be involved in the targeting of profilin and actin at specific intracellular locations. Two other domains that bind proline-rich ligands are SH3 and WW which either localise independent catalytic domains such as kinases and phosphatases to specific intracellular locations or to build multi-component signalling complexes. In contrast the single compact domain of profilin is responsible for both subcellular targeting and functional activity.

Biochemical data suggest that SH3, WW and profilin L-proline binding sequences are similar. This overlap in binding sites may provide a mechanism for the convergence of multiple signaling pathways and so link the

organisation of the actin cytoskeleton to regulatory, developmental and morphogenetic pathways (Mahoney *et al.*, 1997).

3.2.4. Control of Actin Dynamics (See Carlier *et al.*, 1998)

Studies using the two micro-organisms *Listeria monocytogenes* and *Saccharomyces cerevisiae* as models have provided much useful information on the proteins involved in the regulation of F-actin barbed end assembly by cell signalling. Under physiological conditions actin filaments and monomeric actin (ATRG-actin) are maintained in a dynamic steady state referred to as treadmilling. In the absence of regulatory proteins the depolymerisation of the growing (pointed) end is rate limiting compared with the addition of ATRG-actin units to the rapidly polymerising barbed end which is the end of the growing F-actin filament anchored to the plasma membrane. In the absence of regulatory proteins it has been calculated that the net rate of barbed end growth is 0.5sec^{-1} this would allow a lamellipodial protrusion rate of approximately $0.05\text{ }\mu\text{m min}^{-1}$. This is two to three orders of magnitude slower than the observed velocity of actin-based motile processes. To achieve these rates the steady state concentration of ATRG actin should be increased to a few μM which would allow a rate of barbed end growth of $20\text{--}200\text{ subunits sec}^{-1}$. A possible mechanism to achieve this *in vivo* is provided by the cumulative action of capping proteins and actin-depolymerising factors (ADFs)/cofilins. For example gelsolin and other members of that family e.g. scinderin, bind very tightly ($K_A = 10^{11}\text{--}10^{12}\text{M}^{-1}$) to barbed ends and sever filaments. Other weaker non severing capping proteins such as β_2 (CP), bind with an affinity of 10^8 to 10^9M^{-1} . One mechanism by which capping proteins might enhance actin turnover could be the establishment of a high steady state level of ATRG-actin. This would result if the majority of barbed ends were capped. Thus the few barbed ends generated at the plasma membrane in response to signaling would grow very rapidly. This would be due to the funneling of ATRG-actin subunits from the pointed ends of a large number of capped filaments to a selected number of uncapped barbed ends at localised regions of the plasma membrane.

The regulation of barbed end capping is not understood. One hypothesis however is that the signalling cascade leads to phosphatidylinositol 4,5-bisphosphate-elicited dissociation of capping proteins from barbed ends making them available for massive assembly. Some support for this hypothesis is provided by the observation that *in vitro* PIP_2 causes dissociation of CP from barbed ends.

ADF/cofilin proteins are ubiquitous, conserved actin binding proteins which enhance the treadmilling of actin filaments due to the preferential

binding of ADF to the ADP form of G-actin and F-actin. In this way ADF increases the rate of depolymerisation of ADP.F-actin 25-fold, specifically from the pointed end. As a result of the greater flux of depolymerising ADP.G-actin the steady state concentration of ATP.G-actin increases until the steady state rate of barbed end growth balances the high rate of pointed end disassembly.

The mechanisms presented so far in this chapter are all based on the effect of local increases in ATP.G-actin on treadmilling. However a different mechanism has been postulated to account for the rapid turnover of cortical actin appears essential in endocytosis. Of particular interest for this chapter is the report that the activity of ADF/cofilins is regulated by reversible phosphorylation of a serine residue in the amino-terminal region (ser3 in vertebrate ADF; Neb1 *et al.*, 1996). Dephosphorylation of ADF leading to activation is induced by a variety of stimuli (Moon and Drubin, 1995). This results in morphological changes involved in macropinocytotic uptake of hormones or secretion of granules. The regulation of the unknown phosphatase is complex as the phosphatase inhibitors okadaic acid and calyculin A both promote dephosphorylation of cofilin *in vivo* (Takuma *et al.*, 1996; Okada *et al.*, 1996). In order to balance the effect of capping some site-directed nucleation mechanism must operate in conjunction with membrane linked signaling pathways to maintain a constant number of uncapped, steadily growing barbed ends.

3.2.5. The Regulation of Cytoskeleton by PKC

Clearly the growth (balance between F-actin assembly and disassembly) of F-actin chains and its interaction with the plasma membrane is complex. During recent years an increase in our understanding of the regulation of this interaction by kinases has increased. Thus in a recent study (Parente *et al.*, 1999) have found that treatment of secretory epithelia with carbachol and phorbol-12-myristate-13-acetate increased the phosphorylation of a protein (pp66) with 67% homology to the human and bovine coronin-like protein p57. The authors call this protein coronin (se) as it is highly expressed in secretory epithelia. The authors also suggest that coronin (se) plays an important role in the PKC-mediated rearrangements of membrane/cytoskeletal interactions in epithelial cells.

In another study PerezMoreno *et al.* (1998), reported that the sub-cellular distribution of two actin binding proteins vinculin and α -actinin moved towards the cell periphery during Ca^{2+} induced junctional sealing in epithelial cells. In addition they found that vinculin but not α -actinin is phosphorylated by PKC. Several recent studies have focussed on the effect

of phosphorylation on the ERM proteins which link the cell's cortical bilayer to the cytoskeleton. The ERM (ezrin-radixin-moesin) proteins provide these links as their N-terminal halves associate with integral membrane proteins either directly or indirectly via linker molecules such as EBP50 (ERM binding protein, 50 kDa), and their C-terminal domains associate with F-actin. It has now been found that isolated ERM proteins are largely dormant due to an intramolecular interaction between amino- and carboxyl-terminal domains, thus preventing interaction with plasma membrane and F-actin. Simons *et al.* (1998) and Pietromonaco *et al.* (1998) report that C-terminal phosphorylation of Thr⁵⁵⁸ in full length, dormant ezrin and moesin by PKC θ exposes both the F-actin and EBP50 binding sites. Furthermore the phosphorylation of Thr⁵⁵⁸ in moesin increased its association with the cortical F-actin cytoskeleton. In another study Takahashi *et al.* (1999) found that high levels of ezrin were expressed in cutaneous sensory neurones whereas there was little expression in muscle sensory neurones. Thus ezrin might be involved in the formation and/or maintenance of lamina-specific connections for neuronal subpopulations in the visual and somatosensory systems.

The group of Terrian (see Prekeris *et al.*, 1998) have reported an interaction between F-actin and PFC- ϵ . Thus in a recent study Prekeris *et al.* (1998) deleted the putative binding motif in PKC- ϵ (an unique hexapeptide sequence LKKQETaas 222–230 located in the C1 domain) and prevented the binding of PKC- ϵ to F-actin. They also present data to suggest that activation of PKC- ϵ by phorbol esters exposes this F-actin binding site.

In another study Frank *et al.* (1998) provide data to suggest that remodelling of the actin cytoskeleton is regulated by both PKC and the ADP-ribosylation factor nucleotide exchange factor (ARNO). Previous studies have shown that ARNO localises to the plasma membrane *in vivo* and catalyses ARF6 nucleotide exchange *in vitro*. In addition to a role in endocytosis ARF6 has been found to regulate the assembly of the F-actin cytoskeleton. In the present study Frank *et al.* (1998) transfected HeLa cells with ARNO and found that although expression of ARNO led to disassembly of the F-actin stress fibres no obvious change in cell morphology was observed. However treatment of ARNO transfectants with TPA resulted in a dramatic redistribution of ARNO, ARF6 and actin into the membrane protrusions resembling lamellipodia. They also observed that actin rearrangement did not occur in cells expressing catalytically inactive ARNO. PKC phosphorylates ARNO at a site immediately C-terminal to its pleckstrin homology domain. However as mutation of this site had no effect on the ability of ARNO to regulate actin rearrangement it casts doubt on the role of PKC in this process. They also report that an ARNO mutant

lacking the C-terminal PH domain no longer mediates cytoskeletal rearrangement.

Another group of proteins, Cdc42, (LamarcheVane *et al.*, 1998) mediate several signalling pathways leading to actin reorganisation, transcriptional activation and cell cycle control via independent pathways. Using the yeast two-hybrid technique a novel serine- and proline-rich GTPase-activating protein with PKC phosphorylation sites and SH3 binding domains has been isolated. The significance of this observation relates to a) the finding that Rho GTPases play a distinctive role in cytoskeletal reorganisation associated with growth and differentiation, and b) that Cdc42/Rac-binding p21 activated kinase (PAK) and Rho-binding kinases (ROK) act as morphological effectors for these GTPases. In addition the Rho-associated kinase which is activated by small GTP-ase Rho, regulates formation of stress fibres and focal adhesions, myosin fiber organisation and neurite retraction through the phosphorylation of cytoskeletal proteins, including myosin light chain, the ERM family proteins and adducin. Also of relevance is the observation that moesin co-immunoprecipitates with myosin binding subunit. This suggests that MBS is recruited to with moesin to the plasma membrane and that myosin phosphatase and Rho-kinase regulate the phosphorylation state of moesin downstream of rho (Fukata *et al.*, 1998).

In view of our studies implying a role for MARCKS in the regulation of F-actin rearrangement associated with the movement of LDVs to the plasma membrane in SH-SY5Y cells (see later) the study of Matsuoka, *et al.* (1998) is of particular interest. These authors present evidence that phosphorylation of the MARCKS related domain in adducin (a heteromeric protein with subunits containing a COOH-terminal MARCKS related domain that caps and preferentially recruits spectrin to the fast growing ends of F-actin) modifies its *in vivo* and *in vitro* activities. Thus PKC mediated phosphorylation of adducin inhibits actin capping and abolishes the recruitment of spectrin to sides and ends of F-actin. The physiological consequences of the two PKC phosphorylation sites in the MARCKS related domain were investigated using stably transfected MDCK cells with either wild type or S716A/S726A α -adducin mutants which were not phosphorylated by PKC. The adducin mutants did not concentrate at the cell membrane at sites of cell-cell contact but were distributed to the cytoplasm in a punctate pattern. In addition in these mutant cells spectrin was colocalised with α -adducin in the cytosol in a punctate pattern. Immunofluorescence with a phosphoadducin specific antibody detected high levels of phosphoadducin in dendritic spines of hippocampal neurones. Thus adducin is a significant *in vivo* substrate for PKC in a variety of cells and that phosphorylation of adducin occurs in dendritic spines that are believed to

respond to respond to external signals by changes in morphology and reorganisation of the cytoskeleton.

An interesting study which may relate to our recent observation that pretreatment of SH-SY5Y cells with cytochalasin D and colchicine had little effect on potassium or carbachol evoked NA release in the absence of TPA (Danks *et al.*, 1998) is provided by Douglas *et al.* (1997). These authors found that treatment of C6 glioma cells with CD for 1 hr disrupted the F-actin cytoskeleton, increased membrane bound MARCKS from 51 % to 62% yet markedly enhanced the amount of MARCKS translocated to the cytosol following treatment with TPA. In contrast CD had no effect on TPA-enhanced phosphorylation of MARCKS or on translocation of PKC to the plasma membrane. Furthermore the PKC inhibitor bis-indolylmaleimide did not alter MARCKS distribution. Calmodulin antagonists (trifluoperazine, calmidazolium) had little effect on the cellular distribution or phosphorylation of MARCKS but were synergistic with TPA in translocating MARCKS from the membrane without a further increase in its phosphorylation. Thus cytoskeletal integrity is not required for the phosphorylation and translocation of MARCKS in response to activated PKC, but that interaction with both F-actin and calmodulin might serve independently to modulate PKC-regulated localisation and function of MARCKS at cellular membranes.

3.2.6. MARCKS

It follows from the above that although several possibilities may be considered the mechanism by which PKC promotes cytoskeletal rearrangements has not yet been established. In addition to the hypotheses outlined above several studies have suggested that the PKC substrates myristoylated alanine rich C-kinase substrate (MARCKS) and GAP-43 are important proteins in regulating F-actin plasma membrane interactions and possibly exocytosis (Dekker *et al.*, 1991; Coffey *et al.*, 1994; Lui *et al.*, 1994). Similar changes in the actin-based cytoskeleton and phosphorylation of MARCKS underly the phorbol ester-activated release of prolactin from GH₄C₁ cells (Kiley *et al.*, 1992), the secretion of pepsinogen from gastric chief cells (Raufman *et al.*, 1997) glucose-induced insulin secretion (Calle *et al.*, 1992) and of glutamate from hippocampal synaptosomes (Terrian and Ways, 1995). In the non-phosphorylated state MARCKS binds to F-actin and is associated with the plasma membrane (see Blackshear 1993 and Aderem 1995 for reviews). Thus MARCKS is a candidate protein for a role in maintaining the tight F-actin barrier associated with the plasma membrane in chromaffin cells (Trifaro and Vitale, 1993) and SH-SY5Y cells (Goodall *et al.*, 1997a; Danks *et al.* 1999). MARCKS migrates anomalously on SDS gels

with M_r ranging from 60,000 to 87,000 although the primary sequence indicates an M_r value of 31,600Da for the human protein (Harlan *et al.*, 1991). It has a highly conserved N-terminal region and an effector domain of 25 amino acids, referred to as the phosphorylation site domain (PSD) which also contains calmodulin and F-actin binding sites (Graff *et al.*, 1989; Hartwig *et al.*, 1992). Another interesting property of MARCKS which it shares with a second PKC substrate, GAP-43, is its stability to boiling in the absence of SDS which helps to identify and isolate these proteins.

MARCKS can be phosphorylated both in whole cells and cell free extracts with a stoichiometry of 3 or 4 phosphates (depending on species) on serines all of which are located in the PSD (Manenti *et al.*, 1992). Recent studies using recombinant His₆-MARCKS and PKC subtypes expressed and purified from baculovirus-infected insect cells found that the serine residues in the PSD were phosphorylated in a definite order; $S_{156} \Rightarrow S_{163} \gg S_{152}$, although there was little difference in the overall phosphorylation of MARCKS by PKC- β_1 , - δ and - ϵ (Herget *et al.*, 1995). In addition, although PKC- ζ phosphorylated a peptide corresponding to the PSD in MARCKS, no phosphorylation of histone H1S, His₆-MARCKS or GST-MARCKS could be observed (Herget *et al.*, 1995). Phosphorylation results in the dissociation of MARCKS from the plasma membrane and in the loss of its ability to cross-link F-actin (Thelen *et al.*, 1991). For example recent studies in fibroblasts have shown that PKC-dependent phosphorylation regulates the movement of MARCKS between the plasma membrane and Lamp-1 positive lysosomes (Allen and Aderem, 1995). A similar phosphorylation and movement of MARCKS could lead to a selective, partial disassembly of the F-actin cytoskeleton and hence the migration of secretory vesicles to release sites on the plasma membrane in chromaffin and SH-SY5Y cells. Furthermore it has been shown that the related protein MacMARCKS is associated with secretory vesicles in PC12 cells and SSVs in cerebrocortical synaptosomes (Chang *et al.*, 1996). Further support for a role for MARCKS in the regulation of secretion by PKC is provided by studies on the release of ACTH release from pituitary cells by AVP (Lui *et al.*, 1994). Exposure of cultured ovine anterior pituitary cells to 1 μ M AVP resulted in the rapid phosphorylation of MARCKS reaching a maximum plateau by 30 secs. In the same cells AVP stimulated ACTH release biphasically. During the first 30secs there was a rapid burst of ACTH release followed by a slower sustained secretion. The authors suggest, from the correlation between MARCKS phosphorylation and ACTH secretion, that MARCKS phosphorylation may be involved in the initial events associated with secretion of this hormone. An alternative possibility is that MARCKS phosphorylation represents a priming step for the slower sustained phase of

secretion. Thus there are several observations which provide additional support for the suggestion that the MARCKS family of proteins play a role in integrating Ca^{2+} and PKC-dependent signals in the regulation of neurosecretion.

Recent studies (Ohmitsu *et al.*, 1999; Yamauchi *et al.*, 1998) suggest that MARCKS is also phosphorylated by proline-directed protein kinase such as microtubule-associated protein (MAP) kinase and cycline-dependent protein kinase (Cdk5). A mass spectroscopic analysis of intact MARCKS purified from bovine brain revealed at least 6 phosphorylation sites in the N-terminal domain which were upstream of the phosphorylation sites for PKC (Taniguchi *et al.*, 1994). In addition Yamauchi *et al.* 1997b; 1998 found additional phosphorylation sites in the C-terminal region of rat MARCKS. Thus Ser residues in both the N-terminal and C-terminal regions (Ser²⁹¹ and Ser²⁹⁹ in rat MARCKS) have been shown to be followed immediately by prolines. More recently Ohmitsu *et al.* (1999) reported that glutamate acting at the NMDA receptor induced a long lasting phosphorylation of MARCKS in primary cultures of rat hippocampal neurones. Unexpectedly this phosphorylation was not inhibited by the PKC inhibitor calphostin or by down regulation of PKC with phorbol esters but was inhibited by the MAP kinase inhibitor PD 098059. This study also found that glutamate induced a rapid, transient phosphorylation of MARCKS which was inhibited by calphostin. Of particular interest in the study of Ohmitsu *et al.* (1999) is their observation that whereas PKC induced phosphorylation of MARCKS prevented the binding of calmodulin to MARCKS MAP kinase induced phosphorylation had little effect on the interaction between calmodulin and MARCKS. In contrast the introduction of phosphates into MARCKS by MAP kinase prevented the interaction between MARCKS and F-actin to the same extent as PKC induced phosphorylations. Thus PKC is not the only kinase that phosphorylates MARCKS which casts doubt on the use of MARCKS as a specific marker for PKC activation when cells are stimulated with various ligands. However as Schonwasser *et al.* (1996) reported the Ser 113 immediately adjacent to proline in murine MARCKS was not phosphorylated by TPA or platelet derived growth factor in Swiss 3T3 cells the ability of MAP kinase to phosphorylate MARCKS may be limited to only certain cell types. It remains to be determined whether MARCKS in SH-SY5Y cells can be phosphorylated by MAP kinase. Of interest also are reports (Schmitz *et al.*, 1998) that poly ADP-ribose (formed in parallel to DNA strand breaks) binds strongly to the MARCKS family of proteins and inhibits PKC induced phosphorylation of and calmodulin binding to MARCKS. Thus MARCKS proteins and actin could be targets of the poly (ADP-ribose) DNA damage signal pathway.

3.2.7. GAP-43

Recent studies on GAP-43 (B-50, Fl, pp46 or P-57) have clarified its role in regulating the growth state of axonal terminals. Phosphorylation of GAP-43 by PKC appears to be involved in transducing intra- and extracellular signals to regulate cytoskeletal organisation in the nerve ending both in relation to nerve-terminal sprouting and long-term *potentiation* (see Benowitz and Routtenberg 1997 for a recent review). GAP-43 has also been implicated in exocytosis. This protein, which is located on the inner leaflet of the plasma membrane, has been found to become phosphorylated during release of NA from rat hippocampal slices and rat brain cortical synaptosomes (Dekker *et al.*, 1991; Hens *et al.*, 1993a). Hens *et al.* (1993b) have also found that inhibition of PKC activity using the synthetic peptide, PKC_{19–36}, and down regulation of PKC by TPA did not affect Ca²⁺-evoked NA release in streptolysin O permeabilised synaptosomes although these treatments inhibited phosphorylation of GAP-43. These observations suggest that GAP-43 is involved in the processing of vesicles prior to the Ca²⁺ trigger rather than in exocytotic events subsequent to the increase in [Ca²⁺]_i. One possibility is that GAP-43 acts as a calmodulin store since phosphorylation of GAP-43 has been found to decrease its binding to calmodulin. Furthermore Hens *et al.* (1995) report that the monoclonal antibody NM2, directed towards the N-terminal residues 3943 of rat GAP-43 dose-dependently inhibited Ca²⁺-induced NA release from streptolysin O-permeabilised synaptosomes. In contrast antibody NM6, directed against the C-terminal residues 132–213, was without effect. In addition NM2 inhibited PKC-dependent phosphorylation of Ser41 and the binding of calmodulin in permeated synaptosomes. These observations suggest that the N-terminal residues 39–43 of rat GAP-43 play an important role in Ca²⁺-dependent NA release, presumably by serving as a local calmodulin store. Thus a consequence of phosphorylation of GAP-43 is that intracellular levels of calmodulin would increase leading to an activation of CAM kinase II and the subsequent phosphorylation of synapsin I (De Camilli *et al.*, 1990). This could lead to an increase in the number of SSVs at release sites and hence an enhancement of secretion. Support for the involvement of GAP-43 in catecholamine release is also provided by the study of Ivins *et al.* (1993) who reported that isolation of stably transfected clones of PC12 cells expressing GAP-43 cDNA in the antisense orientation had decreased depolarisation-evoked dopamine release. However in similar clones of SH-SY5Y prepared by Dr R Neve (Mailman Research Centre, Harvard Medical School) in which GAP-43 was either over expressed or synthesis decreased, (as detected by western blots) no effect on depolarisation—or muscarinic M₃—evoked release of NA could be observed (PFTV

unpublished observations). Thus the phosphorylation of GAP-43 does not appear to act as a regulator of NA release in SH-SY5Y cells.

3.2.8. Role of MARCKS in PKC Enhancement of Secretion in SH-SY5Y

A possible insight into the mechanism by which protein kinase C could enhance exocytosis has been provided by recent studies using SH-SY5Y. Pretreatment of SH-SY5Y cell layers with TPA for 10 minutes increased the incorporation of $^{32}\text{P}_i$ into two proteins with apparent molecular weights (76 and 46kDa) and isoelectric points ($\text{pI} = 4.0$ and 3.7) corresponding to MARCKS and GAP-43 respectively. The identity of these phosphorylated proteins was confirmed using western blots. Thus both MARCKS and GAP-43 became phosphorylated during the time course of TPA enhancement of NA release. In addition, using a combination of electron microscopy, immunocytochemistry and cell fractionation the following changes were observed (Goodall *et al.*, 1997a; Danks *et al.*, 1999). 1. PKC- α migrated from the cytosol to the plasma membrane. 2. The number of LDVs in a 100nm zone adjacent to the plasma membrane increased 3 fold. 3. MARCKS became phosphorylated and approximately 50% of MARCKS was relocated from membrane to cytosolic fractions. Under these conditions although GAP-43 became phosphorylated no redistribution between cytosol and membrane fractions was observed. 4. Confocal microscopy using fluorescein isothiocyanate labelled phalloidin showed that the F-actin cytoskeleton, which forms a tight barrier at the plasma membrane, partially disassembled.

Furthermore, translocation of MARCKS from the plasma membrane to the cytosol is correlated with the presence of PKC- α (Goodall *et al.*, 1997a). Thus pretreatment of SH-SY5Y cells with the phorbol ester PDBu for 2 hr (conditions which lead to the loss of 40% immunostaining for PKC- α but no decrease of PKC- ϵ immunostaining; (Turner *et al.*, 1996) resulted in a 40% decrease in the translocation of MARCKS to the cytosol induced by TPA (Goodall *et al.*, 1997a). Also, treatment with PDBu for 48 hr, which leads to the down regulation of PKC- α and PKC- ϵ by 90% and 50% respectively (Turner *et al.*, 1996) completely inhibited the translocation of MARCKS. Interestingly phosphorylation of MARCKS was only decreased by about 40% following TPA treatment of cells exposed to PDBu for 48 hr (Goodall *et al.*, 1997a). In view of the recent studies showing that MARCKS can be phosphorylated by kinases other than PKC these may account for the phosphorylation sites in MARCKS which are not affected by down regulation of PKC. In this connection it is relevant that TPA has been reported to stimulate MAP kinase as well as PKC in hippocampal neurones (Kurino

et al., 1995). Thus our recent studies suggest that, in intact SH-SY5Y cells, PKC- ϵ and ζ are unable to phosphorylate all the serines in the PSD of MARCKS. In addition our studies are in agreement with the previous report that all the serine residues in the PSD of MARCKS need to be phosphorylated before translocation can take place (Thelen *et al.*, 1991).

These observations have suggested the following working hypothesis to account for the enhancement of NA release by phorbol esters in SH-SY5Y cells. The initial step is migration of the PKC- α subtype to the plasma membrane. Our previous studies (Turner *et al.*, 1996) have found that NA release is enhanced by PKC- α rather than either of the other two PKC subtypes (PKC- ϵ and ζ) present in SH-SY5Y cells (Leli *et al.*, 1993; Turner *et al.*, 1994; Parrow *et al.*, 1995). A consequence of migration of PKC- α to the plasma membrane is that serines in the PSD of MARCKS are phosphorylated, resulting in a dissociation of the F-actin filaments cross-linked to the PSD (Thelen *et al.*, 1991). This leads to dissociation of a pool of MARCKS from the plasma membrane and disruption of the links between MARCKS and F-actin filaments, resulting in the partial breakdown of the actin cytoskeletal barrier. The loss of this barrier leads to an increase in the number of LDVs adjacent to release sites so that following elevation of $[Ca^{2+}]_i$ NA release is enhanced. Our proposed mechanism could also account for the action of phorbol esters on chromaffin cell F-actin disassembly (Vitale *et al.*, 1992). Our studies also suggest that although all of the serine residues in the PSD of MARCKS need to be phosphorylated for translocation to occur, some of these serine residues are selectively phosphorylated by PKC- α in intact cells. Thus under the conditions used to enhance NA release PKC- ϵ is unable to phosphorylate sufficient serines for translocation of MARCKS to occur.

3.3. Involvement of Isoprenylation/Carboxymethylation in Regulated Exocytosis

3.3.1. Role of Rab3 and Helper Proteins in Controlled Exocytosis

G proteins have been shown to regulate an assortment of cellular processes ranging from nuclear import to endocytotic and exocytotic membrane traffic, maintenance of organelle integrity, assembly of vesicle coat proteins, expansion of ER, vesiculation of the Golgi, regulation of the cytoskeleton, activation of lipases and control of transcription (Sander and Valencia, 1995; Garcia-Pena and Valencia, 1998). In exocytotic processes continuous generation of transport vesicles and regulation of the targeting towards and fusion of these vesicles with the appropriate membranes are a prerequisite. In this sophisticated machinery at least two classes of proteins

do intervene, first the proteins of the SNARE complex and second proteins member of the small G protein superfamily. All low molecular weight (LMW) G proteins share conserved domains important for GTP-binding (Bourne *et al.*, 1991) and hydrolysis implying a cycle between a GDP-bound inactive and a GTP-associated active form (Bourne *et al.*, 1990). The action of bacterial elongation factors and eukaryotic initiation factors in protein synthesis as well as of the α -subunit of heterotrimeric G proteins involved in signal transduction is also based on this GDP/GTP exchange. The small G protein superfamily can be subdivided into four groups including the Ras, the Rho, the Rab and other groups.

The Ras-superfamily has a regulatory assignment in gene expression e.g. through the MAP kinase cascade (Vojtek and Der, 1998). The Rho group is also involved in the regulation of gene expression but additionally a role in the cytoskeleton reorganization is reserved for these G proteins (MacKay and Hall, 1998). The Rab proteins, the most abundant group (over 30 Rab proteins have been described), have been identified as key players in intracellular vesicle transport including exocytosis, endocytosis and transcytosis (Martinez and Goud, 1998). As such, these Rab proteins are localized in the different organelles involved in these specific transport processes suggesting that a unique Rab protein is required for each type of vesicular transport (ER $\rightarrow$ Golgi; Golgi $\rightarrow$ plasma membrane; intra Golgi transport; plasma membrane $\rightarrow$ early endosomes $\rightarrow$ late endosomes; etc.) and even more for each substep. Like ARF Rab's seem also to be involved in vesicle budding (Pfeffer, 1994). This chapter will mainly focus on the Rab3 subfamily comprising the isoforms Rab3A, B, C and D. Rab3 proteins regulate exocytotic vesicle traffic to the plasma membrane in neurons and many secretory cells. Rab3A is mainly expressed in neurons and neuroendocrine cells: it is present in SV membranes (Fischer von Mollard *et al.*, 1990) of neurons and secretory granules (SG) of chromaffin cells (Darchen *et al.*, 1990). Rab3B is localized in secretory granules in the anterior pituitary and lactotroph cells (Lledo *et al.*, 1993; Weber *et al.*, 1994). The ARF family, a member of the "other groups", is also concerned with intracellular vesicle trafficking having a function in vesicle budding from Golgi cisternae and probably many other membrane compartments (Moss and Vaughan, 1998).

Although first evidence of a pivotal role in vesicular transport processes for Rab proteins was found in yeast (Stenmark *et al.*, 1994), it became soon clear that this protein class is also present in mammalian cells and even in a larger number. This correlates well with the diversity of transport routes present in various cell types. Most of the Rab proteins are ubiquitous in mammalian tissues, although expressed to a different level and displaying a differential distribution (Martinez and Goud, 1998). Indeed some of these G proteins are shown to be cell-type specific, regulating

transport events not shared by all mammalian cells; e.g. Rab3A which is only expressed in neurons, exocrine and endocrine cells displaying regulated exocytosis (Fischer von Mollard *et al.*, 1990; Darchen *et al.*, 1990) and not in cells with constitutive secretion as executed by lymphocytes and hepatocytes (Sasaki *et al.*, 1997). Rab3A was detected in adrenal medulla by Darchen *et al.* (1990), where it was specifically expressed in the catecholamine-secretory chromaffin cells. Rab17 can only be traced in epithelial cells (Lutcke *et al.*, 1993). Before, Daucet *et al.* (1989) had identified various LMW GTP binding proteins in cytosolic and membrane enriched subcellular fractions of chromaffin cells from which two major and two minor components are associated with the chromaffin granule membrane.

As already mentioned the Rab protein conformation oscillates between an active GTP-bound membrane associated on-state and an inactive GDP bound off-form located in the cytosol (Bourne *et al.*, 1991). In chromaffin cells it is suggested that about 30% of Rab3A is cytosolic while particulate Rab3A is associated with the chromaffin granule membranes and likely also with the plasma membrane (Aunis *et al.*, 1988). In this "on"- and "off-switching" Rab cycle the intermediary of a number of auxiliary regulatory proteins is necessary. At first, GDI has been postulated to have a role in regulatory GDP/GTP-exchange of Rab3A and its reversible binding to membranes (Sasaki *et al.*, 1997). Moreover GDI has been reported to be active with all members of the Rab family. Rab-GDI (GDP dissociation inhibitor; in fact a family counting at least three members Rab-GDI α , - β and - γ) is a cytosolic protein, which can dislodge the GDP-loaded form (no interaction with GTP-loaded Rab3A) from the vesicular target membrane and translocate it back to the cytosol, where it resides as a GDP-Rab3A-GDI complex waiting for reconversion to the active GTP-bound form. This ternary complex is unable to associate with vesicular target membranes. The model introduced by Sasaki *et al.* (1997) predicts a preliminary dissociation of GDI from the ternary complex, whereafter GTP/GDP-exchange and vesicle association can occur. According to Dirac-Svejstrup (1997) GDI-retrieval is facilitated by the action of a GDI-displacement factor. Such a factor was found to be present in endosomal membranes, able to convert the GDP-bound form of Rab5 and Rab9 complexed with Rab-GDI to the GTP-bound state (Dirac-Svejstrup *et al.*, 1997). A similar factor, although not yet identified, may operate in the Rab3A system. Helper proteins interacting with higher numbered Rab's have also been referred to in the literature. Rabaptin interacts with Rab5 and plays a role in homotypic fusion of early endosomes. Recently an interaction between rabphilin3 free of GTP-Rab3A and rabaptin5 has been reported suggesting that rabphilin regulates endocytosis after rabphilin3 complexed with GTP-Rab3 regulated

exocytosis (Ohya *et al.*, 1998). Other's as the Rab8 interacting proteins are kinases, while Rab6 interacting proteins belong to the kinesin family.

Isoprenylation of Rab3A essential for membrane association is also of crucial importance for its interaction with cytosolic GDI as well as with the other regulatory proteins. GDI indeed is inactive on lipid unmodified Rab. The prenylated tail probably is hidden in a hydrophobic pocket of GDI preventing interaction of Rab3A with the hydrophobic core of the lipid bilayer in the vesicular target membranes as long as Rab is complexed with GDI (Sasaki *et al.*, 1997). A second class of regulatory proteins involved in the Rab3A cycle are the so-called GEP's or GEF's (guanine nucleotide exchange protein factors), which can be recovered from the cytosol and catalyze the GDP/GTP exchange in Rab3A [MSS4 and Rab3A-GRF (guanine nucleotide releasing factor)] (Burnstein *et al.*, 1992; Burton *et al.*, 1993). MSS4 was shown to be also active on other Rab family members (Burton *et al.*, 1993); the substrate specificity of Rab3A-GRF has been less characterized. Takai's group (Sasaki *et al.*, 1997) purified to a high degree from the soluble synaptic fraction two Rab3A specific GEP's respectively GEP I and GEP II, displaying preference for the isoforms Rab3A, -C and -D (inactive on Rab3B and other Rab members). Lipid modification of Rab3A seems to be essential for interaction with GEP's, although some confusing data can be traced in the literature. GEP I and II are only active on the lipidated form of Rab3A, their action being inhibited by GDI. On the contrary MSS4 was equally active on both lipid-modified and unmodified forms; however the inhibitory effect of Rab-GDI required the isoprenylated Rab3A form. After reactivation by GEP the Rab3A protein is transported to the vesicular target membranes, although it has been reported that the nucleotide exchange is not a prerequisite for membrane binding. The GDP/GTP switch is suggested to render the protein resistant to removal by Rab-GDI.

For specific topological association of GTP-loaded Rab3A with the vesicular membranes the presence of a specific protein on the secretory vesicles has been inferred. Such a potential downstream target protein for Rab3A has been isolated in mammals and was Christianized rabphilin3A (Sasaki *et al.*, 1997). Interestingly not only rabphilin but also calmodulin interacts with Rab3A and dissociates the G proteins from synaptic membranes providing a possible link between Ca^{++} - and GTP-dependent regulation of endocytosis. This third type of helper protein in the Rab cycle has shown to bind specifically to the GTP-bound form of Rab3A (and the other Rab3 members) at its N-terminus and maintains Rab3A in its active GTP-bound state by preventing GAP-induced GTP hydrolysis. The C-terminal sequence of rabphilin contains two C2-like domains as found in protein kinase C and also in synaptotagmin, Ca^{++} - and phospholipid (especially

phosphatidyl serine) binding sites. Rabphilin3A itself has no transmembrane sequence and is assumed to be recruited by Rab3A and to interact with the vesicular membrane through the assistance of a not yet identified anchoring protein. The specific localisation of this anchoring protein via its interaction with rabphilin3A is supposed to be decisive for the exact topographic incorporation of GTP-loaded Rab3A in the membrane of the secretory vesicles. This model also predicts that both Rab3A and rabphilin dissociate from the secretory vesicles after GTP hydrolysis. The Rab3A-Rabphilin system is suggested to be a upstream regulator of the NSF-SNAP-SNARE-complex in view of its role in transporting the secretion vesicle to the plasma membrane and as a putative Ca^{++} -sensor (Lian *et al.*, 1994; Sogaard *et al.*, 1994). Interaction of rabphilin3A complexed with GTP bound Rab3A and anchored via its N-terminal on the secretory vesicle with a acceptor protein on the target plasma membrane causes the (pre)docking of the secretion granule in the plasma membrane tentatively followed by synaptotagmin-syntaxin interaction. As so nicely and philosophically verbalized by Geppert and Südhof (1998) Rab3 and synaptotagmin are respectively the “yin” and the “yan” of membrane fusion. The rabphilin system is thought not be involved in the acute fusion process.

Overexpression of full-length rabphilin, but not of deletion mutants lacking the C2 domains, resulted in an increase of catecholamine secretion by chromaffin cells (Chung *et al.*, 1995). On the other hand the Rab3A binding sites of rabphilin are not essential for correct targetting or for the stimulatory function in chromaffin cells (Chung *et al.*, 1997). This observation questions the physiological importance of Rab3A-rabphilin3A interaction in exocytosis; as an alternative explanation it could be that dissociation of the Rab3A-rabphilin complex is imperative for exocytosis to occur. Recently, α -actinin (Kato *et al.*, 1996) has been identified as a rabphilin3A interacting molecule. This interaction, enhancing actin filament cross linking and bundling of α -actinin, can be inhibited by GTP-Rab3A, emphasizing that the Rab3A-rabphilin3A complex is likely to be involved in the organization of the actin filaments during Ca^{++} -dependent exocytosis and opens perspectives that Rab3A and rabphilin3A on the secretion vesicles operate during predocking steps as regulators of the cytoskeleton. As a conclusion the function of rabphilin on secretory vesicles is far from being completely unravelled and waits further elucidation.

Lately (Wang *et al.*, 1997) a novel Rab3-interacting molecule (RIM) was identified, which was absent from secretion vesicles but enriched on synaptic plasma membranes. From its restricted localisation it was implied that RIM can only interact with Rab3 on SV when the SV's have moved close to the active zone and dock, supporting a function for Rab3 in a later stage of exocytosis. Therefore in the present-day scheme two populations

of Rab3 proteins are hypothesized, one for recruitment of rabphilin and the other for binding RIM after docking (Geppert and Südhof, 1998). Following membrane fusion and GTP hydrolysis both rabphilin and RIM disentangle from Rab3, which subsequently dissociates from SV by the action of GDI.

Finally to shut down the Rab effect bound GTP is hydrolyzed by the very low intrinsic GTPase activity of the Rab protein tempting to suggest the involvement of a fourth type of assisting proteins, the GAP proteins (GTPase activating proteins) in order to amplify the GTP hydrolysis. Rab-GTPase seems to act as a docking timer (Lazar *et al.*, 1997; Schimmöller *et al.*, 1998). At least the data for Rab5 involved in endosome fusion are rigorous and convincing (Stenmark *et al.*, 1994). In this respect it is interesting that this Rab5 displays the highest intrinsic GTPase rate. The exact timing of the Rab3A-GTPase activity has not yet been unambiguously established i.e. before, during or after fusion of the secretion vesicles with the plasma membrane. However in heterotypic processes it should make little sense to inactivate a Rab prior to vesicle docking and fusion: once a secretion vesicle generated, it should migrate to the target membrane and fuse. Up till now only one mammalian Rab-GTPase has been characterized. The heterodimeric Rab3A-GAP acts on all Rab3 isoforms, but is inactive towards other Rab members of this family e.g. Rab₂, Rab₅ and Rab₁₁ (Sasaki *et al.*, 1997). As most of the other helper proteins participating in the Rab-cycle also GAP's are inactive on the non-prenylated form of Rab3A. Moreover GAP's are only active on the GTP-bound form of Rab3A delivered from the rabphilin3A complex (Sasaki *et al.*, 1997).

As a conclusion it seems that GEP and GAP proteins determine which guanine nucleotide is bound to Rab on the membrane, in this way modulating the accessibility of Rab to GDI and keeping the Rab's in a dynamic equilibrium between the cytosol and the target membranes. The observation that Rab3-GEP and Rab3-GAP are colocalized with Rab3A at the synaptic release sites suggest that they regulate the activity of Rab3A and are involved in Ca⁺⁺-dependent exocytosis (Oishi *et al.*, 1998).

Evidence for an important role reserved for Rab's in regulated exocytosis has arisen from numerous experimental studies with neuronal and secretory cells including chromaffin cells, perturbing Rab3A metabolism (Lledo *et al.*, 1994). Despite this abundance the role of Rab proteins still is not fully understood. Extremely puzzling results prevent the drawing of a simple clear-cut picture (Aunis, 1998). In chromaffin cells expression of mutant Rab3A proteins and inhibition of Rab3A synthesis modify agonist elicited response by respective inhibition or stimulation of secretion. In this respect it cannot be excluded that the divergent observations may result from how the cells were triggered either by an agonist or e.g. by K⁺-induced

depolarisation (Holz *et al.*, 1994; Johannes *et al.*, 1994). Infusion of purified Rab3A into chromaffin cells delays the onset of exocytosis following depolarisation, while opposite effects have been reported using some patch pipette strategy with rat pituitary lactotroph cells containing Rab3B. From additional data (Weber *et al.*, 1996) it was concluded that the isoforms Rab3A and Rab3B are functionally distinct monomeric GTPases with Rab3B stimulating a late step in Ca^{++} -evoked secretion (when expressed in PC12 cells). Injection of peptides mimicking the Rab3 domain sequence, into secretory cells including chromaffin cells gave rise to diverging results, difficult to interpret because of the variety of side effects these peptides may elicit. A most surprising finding was that mice lacking Rab3A, the major Rab3 isoform, were totally viable, healthy and fertile (Geppert *et al.* 1994). These transgenic mice only showed an increased rate of synaptic depression after repetitive stimulation inferring that Rab3A is non essential for SV exocytosis but merely involved in harvesting vesicles for secretion during repetitive stimulation. A similar observation was made during studies of Rab3 in *C. elegans* demonstrating only mild behavioral deficiency in null mutants (Jorgensen, 1995). While rabphilin3A levels are also decreased by about 70% (emphasizing once more the close partnership between Rab3A and rabphilin3A), the other Rab members are expressed normally. Therefore the possibility has to be considered that some compromised step(s) is (are) compensated by other Rab isoforms. Such reasoning might reconcile the contradictory results from transgenic animals with those on isolated cells. On the other hand in knockout mice no other Rab3 isoforms could be detected in some of the synapses studied, suggesting that Rab3A is not essential for SV docking and fusion despite the fact that it is far the most abundant Rab protein in the nervous and neuroendocrine systems. Triggering exocytosis leads to dissociation of Rab3A from the SV membranes. From toxin experiments with respectively botulinum tetanus toxins (allow Ca^{++} -influx, but block exocytosis) and alatrotoxin (stimulates only membrane fusion) (Stahl *et al.*, 1995; Sudhof and Jahn, 1995) it was concluded that Rab3A dissociation requires both Ca^{++} -influx and triggered membrane fusion, suggesting an action of the Rab3A protein in a late step of exocytosis associated with the membrane fusion process.

Therefore recent theories hypothesize that Rab3A is not essential for docking, priming or fusion (instead of a early function in docking as in most current models), but that Rab3A function becomes manifest in a late step of exocytosis merely involved in regulating neurotransmitter or hormonal release. The experimental data indicate that Rab3A may normally participate in setting the gain of the exocytotic process, by varying the release response per Ca^{++} -influx (Bean and Scheller, 1997).

Finally it must be mentioned that besides Rab's other members of the monomeric GTP binding protein superfamily as well as heterotrimeric G proteins are implicated in regulated exocytosis. Indeed exocytosis can be divided into two steps: a proximal one controlling the level of secondary messengers and a distal step, the exocytotic machinery itself. It was shown that in the former oligomeric G proteins are involved, in the latter LMW GTPases. The $G_{\alpha o}$ subunit together with the $\beta\gamma$ -subunit (Aunis, 1998) and the satellite proteins Rho (Mackay *et al.*, 1998) and ARF (Moss and Vaughan, 1998) probably play a role in both the onset and termination of the exocytotic process. The presence of heterotrimeric G proteins in chromaffin cells as well as their involvement in regulated exocytosis were reported by Knight and Baker (1985). Toutant *et al.* (1987) described a dual localisation in both plasma membranes and granular membranes. The regulation of exocytosis in chromaffin cells by heterotrimeric G proteins is a complex phenomenon e.g. by the fact that at least two heterotrimeric G proteins act in series. Rho, ARF6, Rac and G_o are considered to be putative regulatory elements of the cytoskeleton and granule/cytoskeleton interaction. The $G_{\beta\gamma}$ may have its own function. $G_{\beta\gamma}$ dimers can interact with $G_{\alpha o}$, Rho, Rac and ARF modulating the interactions of these factors with phospholipases and probably polyphosphoinositide phosphodiesterases (Morris *et al.*, 1996). From this it is speculated that a change in membrane phospholipids may be an important event in the exocytotic fusion process. Not all of these cited G proteins are subject of posttranslational isoprenylations, e.g. ARF6 and Gao become only myristoylated (Kahn *et al.*, 1988; Aunis, 1998). ARF6 has been identified in chromaffin granules. Upon stimulation ARF6 becomes detached from the chromaffin granules and can be recovered in a plasma membrane enriched fraction.

3.3.2. Processing of Proteins through Isoprenylation and Carboxymethylation

The discovery of protein isoprenylation in animal cells originates from studies on the relationship between the mevalonate pathway and cell proliferation. Using compactin or mevinolin, both HMGCoA reductase inhibitors, it was shown that a non sterol mevalonic acid derivative was essential for cell cycling (Casey, 1992). Breakthrough came from a study by Schmidt *et al.* (1984) who reported an incorporation of a significant amount of labeled isoprenoid derived from mevalonic acid into cellular proteins. This lipid modification occurred posttranslationally as no inhibition was observed in the presence of inhibitors of protein synthesis (e.g. cycloheximide). The first specific mammalian protein identified as being prenylated

was the nuclear protein lamin B (Beck *et al.*, 1988; Walda and Glomset, 1988).

Protein lipidation does not occur randomly: isoprenyl conjugation is confined to specific families of proteins. The modifications are not of a single type and can broadly be divided in singly or doubly isoprenylated species. Moreover two isoprenoid moieties, all-trans farnesyl and all-trans geranylgeranyl have been identified as the most common modifications with geranylgeranyl being the most predominant isoprenoid covalently linked to the acceptor proteins (Casey, 1992). Prenylation was first described occurring on proteins with a characteristic CAAX-end motif consisting of an invariant cysteine residu fourth from the carboxyl terminal next to two amino acids and the X-amino acid which determines the chemical nature of the isoprenyl group to be attached. This class contains a diverse group of proteins (Zhang and Casey, 1996) ranging from yeast a-mating factors to mammalian Ras and Ras related small G proteins. Prenylation is not limited to proteins containing the tetrapeptide box described (mono-isoprenylation) but does also occur on other members of the super Ras family with either a -XXCC or a -CXC carboxyl end motif. The latter proteins are all geranylgeranylated at both cysteine residues (doubly isoprenylated) and this class is almost exclusively captured by the Rab subfamily of small GTP binding proteins (Zhang and Casey, 1996).

Three different prenyl protein transferases have been identified and characterized at a molecular level both in mammals and in lower eukaryotes (Gibbs and Oliff, 1997). These three transferases can be classified in two functional classes according to their affinity for the respective amino acid sequences in the acceptor substrate: farnesyl protein transferase (FTase) and geranylgeranyl protein transferase type I (GGTase I), which isoprenylate proteins with a carboxyl terminal CAAX-end motif and geranylgeranyl protein transferase type II (GGTase II) or Rab geranylgeranyl transferase which modifies the proteins ending with either a -CXC or a -XXCC sequence. All three enzymes are found in the soluble cellular fraction (Clark, 1992; Schafer and Rine, 1992; Zhang and Casey, 1996).

FTase and GGTase I are both α/β heterodimeric enzymes and share a common α -subunit responsible for the binding of the isoprenyl donor substrates, the pyrophosphorylated derivatives of farnesol or geranylgeraniol (Gibbs and Oliff, 1997). The β -subunit binds to the appropriate peptide acceptor substrate. The nature of the isoprenoid transferred and the transferase involved in modifying CAAX proteins is determined by the terminal amino acid: proteins ending with X = Ser, Met or Glu are farnesylated, those ending with X = Leu are geranylgeranylated (Hrycyna and Clark, 1993). Although these enzymes are highly specific, the substrate

specificities are not absolute and cross prenylation often occurs (Gibbs and Oliff, 1997).

GGTase II is also a heterodimeric a/b enzyme and displays many common characteristics with the CAAX prenyltransferases. A striking difference is that in addition to the carboxyl terminal amino acid box it requires supplementary information from upstream amino acid sequences (Casey and Seabra, 1996). Before isoprenylation can occur, Rabs must be bound to Rab escort proteins (REPs) (Gelb *et al.*, 1998). Two REPs have been characterized in mammalian cells which bind the Rab protein until fully isoprenylated. After bigeranylgeranylation these REPs assist in guiding the prenylated Rabs to the membranes, nevertheless binding of Rabs by GDI proteins is also reported (Zhang and Casey, 1996).

Following prenylation of the cysteine residu at the CAAX terminus the three C-terminal amino acids are trimmed by the action of a metallo-endoprotease. The processing is dependent on the prenylation status of the substrate with a higher affinity for the isoprenylated peptide substrate. In mammalian systems this proteolytic activity is membrane bound. Recently the membranous enzyme has been biochemically identified, characterized and cloned. The protease should consist of multiple subunits (Nishii *et al.*, 1997). Membrane binding studies of fully processed and partially processed Ras suggest that proteolysis may be important in Ras function (Chen *et al.*, 1996). The Rab series of small GTP binding proteins C-terminating with -XXCC or -CXC are not subject of the endoproteolytic cleavage. Following isoprenylation and endoproteolysis the newly exposed terminal cysteine residu is methylated. The last step of this tripod is catalyzed by a SAM-dependent prenylated protein specific methyltransferase i.e. carboxyl methyltransferase type III (PCMT III) (Clarke *et al.*, 1988, 1992; Hrycyna and Clarke, 1993; Rando, 1996). The methyltransferase shows no preference for either farnesylated or geranylgeranylated proteins (Hrycyna and Clarke, 1993). This methyltransferase requires only a thioalkyl moiety and no additional information from amino acid sequences is required (Rando, 1996). Isoprenylated proteins with a -XXCC carboxyterminal are no subject to methylation. The enzyme has been recently characterized at the molecular level (Dai *et al.*, 1998) and purified from bovine brain (Yao *et al.*, 1998). The mammalian eukaryotic carboxyl methyltransferase is membrane bound although the exact subcellular localisation is not clear (Hrycyna *et al.*, 1993; De Busser *et al.*, 1995; Rando, 1996; Dai *et al.*, 1998;). Long time it had been thought that this methylation reaction is the only one in the overall prenylation process that is potentially reversible and as such suggested as an attractive metabolic regulatory site. Indeed, a methylesterase activity selective for cysteine containing methylesters has been described (Tan and Rando, 1992; Dunten *et al.*, 1995; Van Dessel *et al.*, 1998). This

counterplayer of the methyltransferase is even less well characterized. Recently, Zhang *et al.* (1997) isolated a prenylcysteine lyase from bovine brain. This enzyme hydrolysed the thioether bond between the farnesyl group and the cysteine. The authors suggest that this enzyme is mainly involved in the catabolism of prenylated proteins most likely in the final step of degradation.

The overall strength of the hydrophobic interactions involving farnesylated or geranylgeranylated protein would not dramatically change through addition of a methyl group (Rando, 1996). Nevertheless, Backlund (1992) identified two pools of G25K proteins in rabbit brain. The cytosolic pool in contrast to the membranous pool seemed not to be methylated.

3.3.3. Regulatory Function of Protein Prenylation/Carboxymethylation in Exocytosis and Other Cellular Processes

Functions of Protein Prenylation. How essential is protein isoprenylation for membrane associated and exocytotic processes? Ras proteins require both farnesylation and a C-terminal cluster of basic amino acid residues or palmitoylation for membrane association (Gelb *et al.*, 1998). Recent studies have shown that Ras proteins are functional as long as the hydrophobicity of the prenylgroup is preserved (Gelb *et al.*, 1998). Site-specific mutagenesis substituting a serine residue for the cysteine residue normally isoprenylated resulted into an inactive Ras. On the other hand, mutations that abolished palmitoylation, proteolysis or the polybasic domain only slightly impaired the transformation ability of H-Ras proteins (Lobell, 1998), while lack of palmitoylation of K-Ras 4B resulted in a tenfold reduction of membrane binding and severe impairment of its signalling activity. These transformation studies show that the only processing event that is absolutely essential to the transforming ability of Ras is the farnesylation step. However, it should be stressed that a number of experiments were performed in conditions where the Ras protein was overexpressed as intervening partner. Further proteolysis and methylation seem to be important for the proper targeting and full function.

It is also suggested that Ras prenylation plays a role in protein-protein interactions e.g. yeast Ras 2 regulates adenylate cyclase and the interaction of H-Ras and IK-Ras with SOS (Gelb *et al.*, 1998). In the visual transduction cycle it seems that isoprenylation activates two proteins: a farnesylated kinase (rhodopsin kinase) which is translocated in a reconstituted system to rhodopsin-containing membranes upon photobleaching and the farnesylated γ -subunit of the transducing $\beta\gamma$ -complex which is required for high affinity binding to the α -subunit. Parish and Rando (1996) report that both farnesylation and methylation also contribute to the membrane binding of

this γ -subunit. Evidence for interaction of prenylated proteins with other proteins is only available for Rab proteins (Sasaki *et al.*, 1997). Rab-GDI should contain a binding site for the lipid chains making the complex water soluble. The role of Rab3A protein in exocytosis and the essential role of isoprenylation has been referred to in the previous section. As outlined non isoprenylated Rab proteins do not interact with their upstream partner-proteins. The exact moment and subcellular site of isoprenylation/methylation remains to be elucidated.

Carboxymethylation. Several functions for “extra” methylation of prenylated proteins have been put forward: (i) activation of certain cellular proteins as reported for the mating pheromone α -factor from *S. cerevisiae*, an *in vivo* substrate for the STE 14 methyltransferase (Hrycyna and Clarke, 1993); (ii) a role in efficient membrane attachment guiding the mature protein to the specific target membrane (Backlund, 1992); (iii) involvement in the association with specific receptor proteins at the proper subcellular site (Hrycyna and Clarke, 1993); (iv) protection from proteolytic digestion (Hrycyna and Clarke, 1993). Carboxymethylated isoprenylated proteins display a longer half life time. Methylation eliminates the negative charge at the C terminus of the prenylated proteins increasing its hydrophobicity. Although it seems that in proteins the enhanced membrane affinity is merely due to the neutralisation of the negative charge than to the minor increase in hydrophobicity. Methylation drastically increases ($\times 100$) the n-octanol-water partition coefficients of AFC and to a much lesser extent ($3 \times$) that of AGGC inferring that the role of methylation is more important for farnesylated protein than for the more hydrophobic (di)geranylgeranylated protein (Rando, 1996). In proteins the enhanced membrane affinity is merely due to the neutralisation of the negative charge than to the minor increase in hydrophobicity. The absolute necessity of the methylation seems to be variable depending on the experimental system under investigation.

Carboxymethylation of trimeric vs LMW GTP-binding proteins may play a key role in regulating a variety of cellular processes. Methylation of both the large G proteins (γ -subunit) and small G proteins may be important in the regulation of the chemotactic response in mammalian cells as inferred from the drastic inhibition elicited by AFC, a competitive inhibitor of the intervening methyltransferase (Rando, 1996). AFC has been reported to inhibit platelet aggregation and cell growth (Hazoor-Akbar *et al.*, 1993). The inhibitory effect of AFC and derivatives are most likely not the result of simple membrane perturbation, as the physiologic action seems to be extremely sensitive to small changes in chemical structure.

Rac proteins, another member of the family of Ras related small GTP-binding proteins, are involved in the inflammatory response in macrophages

and neutrophils through the intermediate of the multicomponent NADPH oxidase system (Backlund, 1992). In these cells prenylated but unmethylated G25K proteins could be recovered from the soluble fraction. Upon exposing the Rac protein to the GTP-analogue GTP γ S the Rac protein was guided to the membrane and subsequently methylated. From this it has been hypothesized that GTP bound proteins are more efficiently carboxymethylated than the GDP-loaded species. From the divergent effects reported by Ding *et al.* (1994) for a series of farnesyl-L-cysteine analogues on superoxide release by human neutrophils, the authors conclude that these analogues do not evoke their pharmacological effects via methyltransferase blockade but suggest they interact with a hitherto undefined target protein in the neutrophils. Also according to Perez-Sala *et al.* (1998) the biological effects of farnesyl cysteine analogues appear to be independent from their behaviour as methylation inhibitors. From their finding with a variety of farnesyl analogues they conclude that FTA is a powerful inhibitor of HL-60 cell growth and induces apoptosis, but that neither inhibition of isoprenylated protein methylation nor impairment of Ras membrane association are essential. In this respect an attractive theory is that methylation/demethylation reactions have cross talk with the GDP/GTP exchange mechanism involving GEP's/GAP's in order to regulate the amount of activated G protein associated with the membrane. With regard to the transducing system from their experimental data Rando *et al.* (1996) conclude that farnesylation of the T $_{\beta\gamma}$ subunit appears to be essential while methylation was suspected to be only of quantitative importance. These authors also draw attention to the fact that complex formation of the α and $\beta\gamma$ subunits of transducing are dependent on the state of farnesylation/methylation. While the effect of methylation is minimal in the rhodopsin/transducin system it cannot be excluded that the effect provoked by isoprenylation/methylation is much more explicit in other systems e.g. methylated T $_{\beta\gamma}$ was quite effective in activating PI3 kinase and PLaseC β , while the unmethylated form was nearly inactive. With regard to T $_{\beta\gamma}$ it should be emphasized that the transducing heterotrimeric protein is the only oligomeric G protein that is farnesylated, all others are object of geranylgeranylation. With regard to Rab proteins involved in regulated exocytosis in chromaffin cells geranylgeranylation seems to be of crucial importance for Rab interaction with the regulatory proteins Rab-GDI, Rab3A-GEP and Rab3A-GAP whereas carboxy-methylation has shown not to be essential at least for interactions with GDI. It remains to be established which modifications are critical for the action of GEP and GAP regulatory proteins. Moreover, it is still an open question whether the lipid moieties interact directly with these regulatory proteins or that lipidation

at first induces a conformational change in the Rab3A itself, promoting the interaction with these regulatory proteins. As suggested by Sasaki *et al.* (1997) three-dimensional structural analysis may clarify the function of lipidation in these protein-protein interactions.

Next to regulated exocytosis of catecholamines by chromaffin cells another well characterized endocrine system involving prenylated proteins is the insulin release by pancreatic β cells. Metz *et al.* (1993) have shown that posttranslational modification of GTP-binding proteins influences the glucose induced insulin release. Inhibition of the carboxyl methylation of Cdc42 and Rap1 reduced the nutrient induced secretion. Also results from Li *et al.* (1993) point to the involvement of an isoprenylated protein, most likely belonging to the LMW G family, in the potentiation of nutrient induced insulin secretion. However, phorbol esters and mastoparan induced secretion was not effected neither by inhibition of the prenylation nor by preventing the methylation. From these observations it is suggested that glucose and GTP dependent carboxymethylation of Cdc42 is an obligatory step in the stimulus-secretion coupling of nutrient induced insulin secretion, but not in the exocytotic machinery itself.

Nevertheless these data infer that carboxymethylation of certain G proteins is a prerequisite for the interaction with signalling effector molecules. In the same line Regazzi *et al.* (1995) have demonstrated that prenyl-cysteine analogues stimulate under basal conditions and potentiate insulin secretion by Ca^{++} and GTP γ S, however by a mechanism not related to the inhibition of methyltransferase III. Rap1 in pancreatic β -TC cells displayed enhanced methylation in response to glucose and depolarizing potassium concentration.

The $\text{Na}^+\text{-H}^+$ antiport in human platelets has been shown to have the typical carboxyl terminal tetrapeptide box required for lipid modification by isoprenylation/methylation. Data from Otsuka *et al.* (1998) indicate that inhibition of carboxymethylation reduces basal pH_i and alters the properties of the $\text{Na}^+\text{-H}^+$ antiporter. It is suggested that carboxymethyltransferase is implicated in intracellular pH homeostasis. In this respect Hooley *et al.* (1996) indicate that in CCL 39 fibroblast Rho-like proteins intervene in the regulation of H^+ -exchange and pH_i .

In gastric chief cells Raffaniello and Raufman (1995) showed, using AFC and AGGC as methylation inhibitors, that GTP γ S induced pepsinogen secretion does not require carboxymethylation of LMW GTP-binding proteins. Contrary, Blazer-Yost *et al.* (1997) could demonstrate that methylation of a geranylgeranylated protein was involved in the nitriferic action of aldosterone in polarized epithelial cells. In developing front cerebellum Paz *et al.* (1993) noted a marked coordinated rise of the methyltransferase

and its substrates during the critical period of cerebellar granule cell migration and synaptogenesis suggesting a regulatory role for the enzyme and its target substrates in cerebellar ontogenesis.

4. ENDOCYTOSIS OF LDV/SECRETORY VESICLES

The membrane of the secretory vesicles, after transient exocytotic fusion of the vesicle membrane with the plasma membrane, is rapidly and selectively retrieved back into the cell. While exocytosis requires Ca^{++} and ATP, the subsequent endocytosis can proceed in the virtual absence of Ca^{++} and ATP and is largely unaffected by a variety of nucleotide triphosphates (including nonhydrolyzable analogues) and cyclic nucleotides. While retrieval of SSV-constituents is well documented (for a review see Südhof, 1995), data on the internalization of noradrenergic LDVs and secretory vesicles remain fragmentary. Most of our knowledge concerning recycling of secretory granules/LDVs comes from studies on chromaffin cells, where retrieved secretory granule constituents have been shown to recycle via the *trans*-Golgi network region (Patzak and Winkler, 1986; Philips, 1987; Hurlley, 1993). In noradrenergic neurons in culture it is shown that after stimulation, exocytosed LDV-constituents enter the endocytotic pathway via clathrin-coated pits and clathrin coated vesicles of a relatively smaller size than the LDV (Annaert *et al.*, 1997). In the same neuronal cultures it is demonstrated that LDV-membrane constituents are retrieved into early endosomes after exocytosis, as can be concluded from their colocalisation with the endosomal markers Rab5 and HRP in subcellular fractionation analysis and their colocalisation with Rab5 in confocal microscopy (Partoens *et al.*, 1998). Rab5 is a general specific marker for early endosomes (Chavrier *et al.*, 1990) and its occurrence on synaptic vesicles provided evidence that SSV-membrane constituents recycle via an endosomal intermediate (Fischer von Mollard *et al.*, 1994; de Hoop *et al.*, 1994). Rab5 regulates the early steps of the endocytotic pathway, i.e. internalization at the plasma membrane and homotypic fusion of early endosomes with exchange of contents (Gorvel *et al.*, 1991; Bucci *et al.*, 1992). Therefore, the presence of Rab5 on both retrieved LDV- and SSV-membranes in the stimulated SCG neurons indicates their possible intermixing and the formation of a common sorting endosomal compartment. Interestingly, it was recently reported that Rabphilin3a, previously characterized as a Rab3a-binding protein (Shirataki *et al.*, 1993) and regulator of regulated exocytosis (Holz *et al.*, 1994), interacts with the Rab5-Rabaptin5 system (Ohya *et al.*, 1998). Rab3a, in its GTP-bound state, inhibited the Rabaptin5-Rabphilin3a interaction (Ohya *et al.*, 1998) as well as the promoting effect of Rabphilin3a on

membrane internalisation (Burns *et al.*, 1998). In chromaffin cells the presence of Rab3a/a-d on retrieved secretory vesicle membranes was reported (Slembrouck *et al.*, 1999). The hypothesis can be formed that the Rab3a, present on retrieved secretory granules, competes with Rabaptin5 for interaction with Rabphilin3a thus prohibiting a fusion of retrieved membranes with the common endosomes. Further work has to be done to investigate the role of Rab3a as a regulator for sorting events in granule membrane recycling.

It is reported that a large part of retrieved LDV membranes is supposed to be retrogradely transported to the cell body (for a review, see Thureson-Klein and Klein, 1990). In splenic nerve, this retrograde transport of LDV-membrane constituents occurs via multivesicular bodies (Annaert *et al.*, 1994). However, no information is available on the possible sorting in the endosomal compartment between the retrograde transport pathway and a recycling pathway whereby some membrane constituents are re-utilized for the biogenesis of other storage organelles i.e. the SDVs. In chromaffin cells evidence is provided that at least a part of the retrieved secretory vesicle membranes can rapidly re-enter the secretory cycle (Von Grafenstein and Knight, 1992). Endocytosed vesicles may therefore not have to recycle via the trans-golgi network for the formation of new secretory vesicles in order to re-enter the regulated secretory pathway.

ACKNOWLEDGMENTS. The Laboratory of Neuropharmacology (W.D.P., P.P. and D.S.) is supported by grants from the Queen Elisabeth Medical Foundation and the EC-project (BIOMED 2 Programme) BMH4-CT96-1586. D.S. holds a grant from the IWT (Institute for the encouragement of Scientific and Technological research in the industry).

P.F.T.V. would like to thank The British Heart Foundation, The Medical Research Council and The EU Biomed-2 programme for supporting the work summarised in this chapter which was carried out in his laboratory.

The work of the laboratory of Human Biochemistry (AL, GVD, HDB) is supported by RAFO grants (1LAGRAKP97 and 1VDESGKP98).

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Chapter 10

Reversibility in Fusion Protein Conformational Changes The Intriguing Case of Rhabdovirus-Induced Membrane Fusion

Yves Gaudin

1. GENERAL INTRODUCTION

Enveloped viruses enter cells by protein mediated membrane fusion. Viruses such as paramyxoviruses and retroviruses fuse directly with the plasma membrane. For other viruses such as alpha-, rabdo- and orthomyxoviruses, viral particles are first internalized and then fuse with the endosomal membrane. In both cases, the ectodomain portion of the fusogenic glycoprotein undergoes a major structural rearrangement to generate a fusion competent state which exposes a previously buried hydrophobic peptide which is then able to interact with and destabilize the target and/or viral membranes. For viruses fusing at the plasma membrane, the conformational change is triggered by the interaction with one or more cellular

YVES GAUDIN Laboratoire de Génétique des virus, CNRS, 91198 Gif sur Yvette Cedex, France.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

receptors whereas for viruses fusing in the endosome, the conformational change is triggered by the low pH of this internal compartment. In most of the cases, when the conformational change is triggered in absence of a target membrane, it leads to inactivation of the fusion properties of the viral glycoprotein.

Despite extensive work, mainly on influenza virus, the actual mechanism by which two lipid bilayers fuse remains largely obscure. Strong binding of water to the lipid polar head groups account for a strong, exponentially increasing repulsion when membranes approach each other at distance less than 2nm. This is the so-called hydration force (Rand, 1981). To mediate fusion, viral or cellular fusogenic proteins have to bypass this repulsive force. Experiments performed on model systems (e.g. Lee and Lentz, 1998) but also on virus-induced membrane fusion (Chernomordik *et al.*, 1997; 1998) suggest that the fusion intermediates are very similar from one system to another. The first intermediate seems to be a stalk made by local lipidic connections between contacting monolayers of fusing membranes. The formation of this intermediate is inhibited by inverted cone shaped lipids such as lysophosphatidylcholine and favored by the presence of cone shaped lipids like oleic acid (Chernomordik *et al.*, 1997; 1995). Stalk formation is most probably followed by a local and transient hemifusion diaphragm in which a fusion pore appears and expands (Chernomordik *et al.*, 1998; Kemble *et al.*, 1994). Although estimates of the energetics of formation of these putative fusion intermediates have been made (Siegel, 1993), the exact role of the fusogenic proteins in the formation of these membrane structures is unclear. An increasing number of experiments now suggests that the fusion complex is made up of several fusogenic proteins that act in a concerted manner (Markovic *et al.*, 1998; Danieli *et al.*, 1996; Blumenthal *et al.*, 1996; Plonsky *et al.*, 1996). This number of fusion proteins may help to overcome the activation energy of the fusion process which is in the range of 40 kcal/mol (Lee and Lentz, 1998; Clague *et al.*, 1990). Furthermore, in most cases, the fusogenic protein is synthesized in a metastable conformation and it has been proposed that the energy released during the fusogenic structural transition is used to achieve the energetically expensive membrane-fusion reaction (Ruigrok *et al.*, 1986; Carr *et al.*, 1997). However, there is at least one exception to this metastability rule: for rhabdoviruses, which have low pH-induced fusogenic properties, the low pH-induced structural transition is absolutely reversible. This will be the subject of this chapter.

2. METASTABILITY OF THE NATIVE VIRAL MEMBRANE FUSION GLYCOPROTEIN IS GENERAL

2.1. Influenza HA as the Model Fusogenic Glycoprotein

2.1.1. General

The best characterized fusogenic glycoprotein is influenza hemagglutinin (HA) because detailed structural information on the different conformations of the glycoprotein is available (Chen *et al.*, 1998; Bullough *et al.*, 1994; Weiss *et al.*, 1988; Wilson *et al.*, 1981). HA is synthesized as HA0, a precursor molecule which is endoproteolytically cleaved by a cellular protease to produce a receptor binding domain (HA1) and a membrane-anchored fusion domain (HA2). HA is a homotrimer, each protomer consisting of an HA1 and HA2 chain connected through a single disulfide bridge. The HA ectodomain can be released from the viral membrane after cleavage by bromelain. This soluble form called BHA has been crystallized and its structure has been determined (Wilson *et al.*, 1981). Most of BHA2 forms a fibrous triple coiled coil and HA1 is situated at the top of this coil and contains the antigenic sites (Wiley and Skehel, 1987) and the binding domain for sialic acid (Weiss *et al.*, 1988) which is the viral receptor. At low pH (between pH 5 and 6 depending on the influenza strain), HA undergoes an **irreversible** conformational change (Bullough *et al.*, 1994; Doms *et al.*, 1985; Skehel *et al.*, 1982). During this structural transition, the highly hydrophobic aminoterminal peptide of HA2, the so-called fusion peptide, which is initially buried in the native structure, is exposed. As a consequence, HA is able to bind and destabilize lipid bilayers.

2.1.2. Low pH-Induced HA Conformational Change

Structural studies on the low pH structure of HA revealed one of the biggest conformational change ever detected in a protein. First, low pH treatment results in the loss of HA1-HA2 interactions and dissociation of the HA1-HA1 interface stabilizing the native structure. After the structural transition, the monomeric structure of HA1 which has been determined after crystallization as a complex with a monoclonal antibody Fab fragment is not significantly modified (Bizebard *et al.*, 1995). This is in agreement with previous results indicating that after the low pH structural transition, HA1 is still able to bind the viral receptor (Sauter *et al.*, 1989) and is still recognized by monoclonal antibodies against native HA1 (Bizebard *et al.*, 1995; White and Wilson, 1987; Daniels *et al.*, 1983; Webster *et al.*, 1983; Yewdell *et al.*, 1983). In fact, the low pH-induced conformational change is essentially

a dramatic refolding of HA2 (Figure 1) in which a region folded as a long loop in native HA2 (labeled B in Figure 1) converts to a three-stranded coiled coil resulting in the projection of the aminoterminal fusion peptide 100Å to the end of a long coiled coil rod (Bullough *et al.*, 1994; Carr and Kim, 1993). This is accompanied by a relocation of the carboxyterminal membrane anchor, by a fold back mechanism, to the aminoterminal end of the rod-shaped molecule (Bullough *et al.*, 1994). This orientation of both extremities of HA2 after the low pH-induced structural transition has been confirmed using monoclonal antibodies (Wharton *et al.*, 1995) and is in agreement with results obtained from hydrophobic photolabeling experiments indicating that the fusion peptide inserts into the viral membrane in the absence of target membranes (Weber *et al.*, 1994).

In the absence of a target membrane, the low pH-induced conformational change leads to inactivation of the fusion properties (White *et al.*,

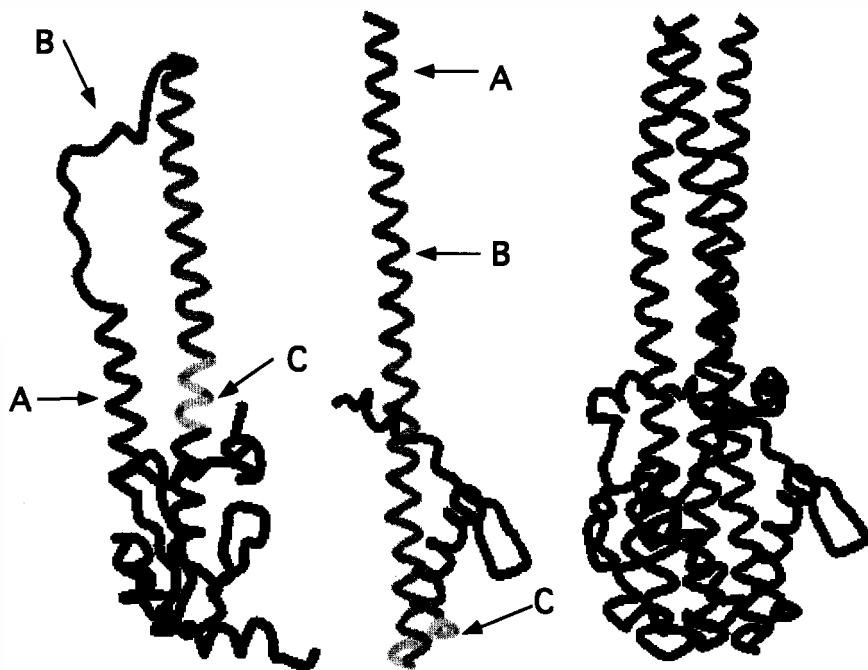


FIGURE 1. Structure of native BHA2 and low pH TBHA2. Left: Native BHA2. Middle: Low pH TBHA2 resulting from cleavage of low pH BHA2 by thermolysin which removes the fusion peptide and the two beta strands located below the helix labeled A. The BHA2 loop (labeled B) is now forming a long helix, the BHA2 helix labeled C is now a connecting loop. Right: trimeric structure of TBHA2. All figures were drawn with program Molscript (Kraulis *et al.*, 1991).

1982). Thus, the X-ray determined low pH structure of HA2 is probably associated with the low pH inactivated form. From this result, there has been a debate on the exact role of this conformational change in the fusion process. A first model has proposed that the fusion-active form is not significantly different from native HA because fusion was detected under conditions where changes in HA shape were not detected either by electron microscopy or by using antibodies (Stegmann *et al.*, 1990; Puri *et al.*, 1990; Stegmann *et al.*, 1987). However, as in these experiments, it was difficult to exclude that the few hemagglutinins that were directly involved in fusion had already undergone their structural transition; a second model proposes that HA2 conformational change is absolutely required for fusion. This view is now largely supported by the following observations:

- (i) In most cases, changes in hemagglutinin conformation and fusion occur at the same pH and temperature (Ruigrok *et al.*, 1986; Wharton *et al.*, 1986).
- (ii) The rate-limiting step in influenza fusion depends on the same process as that leading to inactivation (Ramalho-Santos *et al.*, 1993).
- (iii) In the absence of a target membrane, the HA conformational change leads to insertion of the fusion peptide in the viral membrane (Wharton *et al.*, 1995; Weber *et al.*, 1994).
- (iv) Proline substitutions in the region of high coiled-coil propensity of HA2 have been shown to impair fusion activity (Qiao *et al.*, 1998).
- (v) Many other viral fusion proteins have been shown or are predicted to contain coiled-coil regions (Bagai Joshi *et al.*, 1998; Weissenhorn *et al.*, 1998; 1997; Chan *et al.*, 1997; Fass *et al.*, 1996; Chambers *et al.*, 1990). Furthermore, mutagenesis studies and peptide inhibition have supported the idea that coiled-coil formation is important for fusion (Furuta *et al.*, 1998; Yao and Compans, 1996; Ramsdale *et al.*, 1996; Reitter *et al.*, 1995; Buckland *et al.*, 1992).

2.2. Irreversibility of the Fusogenic Structural Transition Is a Common Feature of Viral Membrane Fusion

2.2.1. The Case of Fusogenic Glycoproteins Activated by Proteolytic Cleavage

Recent structural studies of the fusion glycoprotein of retroviruses (HIV and moloney murine leukemia virus[MLV]) and of Ebola virus have indicated similarities with the structure of TBHA2 (Weissenhorn *et al.*,

1998; 1997; Chan *et al.*, 1997; Fass *et al.*, 1996). A common feature of all these viruses is that, like influenza, their fusion glycoproteins are synthesized as a precursor that must be cleaved to generate the aminoterminal fusion peptide. This cleavage results in gp120 and gp41 for HIV and SIV, SU and TM for Moloney and Gp1 and Gp2 for Ebola. Gp41, TM and Gp2 constitute the transmembrane part of the complete protein and are equivalent to influenza HA2. Their ectodomains form rod-shaped α -helical bundles. In the structural studies, both the carboxyterminal membrane anchor and the aminoterminal fusion peptide were removed (in order to have a soluble molecule) but, as for TBHA2, the results strongly suggest that these peptides are collocated at one end of the rod (Skehel and Wiley, 1998). Differently from HA, the structure of the native complete glycoprotein is not known for any of these viruses and it is probable that the native, complete fusogenic glycoproteins (i.e. the complexes gp120/gp41; SU/TM and Gp1/Gp2) are folded into a conformation that is different from that present in these structures. The fragments used for these studies were expressed in bacteria or chemically synthesized and were shown to fold spontaneously into these rod-shaped α -helical bundles which appear to be extremely resistant to thermal denaturation (Lu *et al.*, 1995). Thus, the structure determined for gp41, TM and Gp2 is supposed to be the most stable conformation of this part of the molecule. As mentioned above, this conformation is not the native conformation but most probably represents the structure of the membrane anchor fragment at the end of the fusion process. Thus, as for HA (Chen *et al.*, 1998), the consequence of the proteolytic cleavage of the fusogenic protein is to activate the potential for triggered conformational change by rendering the native structure metastable.

2.2.2. The Case of Uncleaved Fusogenic Glycoproteins

There are other viral fusogenic glycoproteins that are able to undergo an irreversible conformational change without any activating cleavage. For these proteins, the fusion peptide is supposed to be internal (i.e. not aminoterminal). The best characterized viral glycoproteins belonging to this category are the protein E1 of Semliki forest virus (SFV) and the protein E of tick borne encephalitis virus (TBEV). In both cases, the conformational change results in an irreversible new oligomeric arrangement of the fusion protein.

In the case of SFV, the spike is synthesized as a p62E1 precursor. P62 is then cleaved into P2 and P3 and the native spike is made as a trimer consisting of (E1/E2/E3)₃. Upon exposure to low pH (6.2 or below), E2 and E1 dissociate and this is followed by the formation of a new trimer E1₃ (Kenney *et al.*, 1994; Justman *et al.*, 1993; Wahlberg and Garoff, 1992; Salminen *et al.*,

1992; Wahlberg *et al.*, 1992). In the case of TBEV, E is transported in association with prM which is further cleaved into M (Heinz *et al.*, 1994). The glycoprotein at the viral surface has an elongated dimeric structure (Rey *et al.*, 1995). This dimer has been cleaved to release a soluble ectodomain and this soluble form has been crystallized and the structure has been solved (Rey *et al.*, 1995). This structure is completely different from the BHA structure. Upon acidification, it has been shown that at the viral surface, the dimers dissociate and reoligomerize irreversibly into trimers (Allison *et al.*, 1995).

In both cases, the metastability of the native conformation is the consequence of the activating cleavage which, in these cases, does not occur in the fusion protein but in an associated protein (p62 or prM) which prevents the low pH-induced fusogenic transition.

3. THE RHABDOVIRUS EXCEPTION

3.1. The Rhabdovirus Family

3.1.1. General

Rhabdoviruses are wide-spread among a great diversity of organisms (including plants, insects, fishes, mammals, reptiles and crustaceans). They have in common a bullet-like shape. The two most studied genera of rhabdoviruses are the lyssaviruses (prototype virus: rabies virus [RV]) and the vesiculoviruses (prototype virus: vesicular stomatitis virus [VSV]). Viruses of both genera are composed of a capsid containing a single negative strand of RNA (about 12 kb) associated with three proteins (N, P and L) and condensed in a compact helical structure by the matrix protein (M). This compacted nucleocapsid is surrounded by a membrane which contains a single transmembrane protein G.

3.1.2. The Rhabdovirus Glycoprotein

The rhabdovirus glycoprotein G constitutes the spikes that protrude from the viral surface and that play a critical role during the initial steps of virus infection. First, it initiates the interaction between the virus and the target cell: it is responsible for virus attachment to specific receptors. In the case of VSV, phosphatidylserine (PS) is supposed to play the role of receptor (Schlegel *et al.*, 1983). As PS is an ubiquitary molecule, this would explain the large tropism of VSV. In the case of rabies virus, many molecules have been proposed to play the role of receptors: although rabies virus

is a strict neuropathogen in animals, rabies strains adapted to *in vitro* cell culture have a wide host range and infect nearly all mammalian and avian cell types tested. It has been proposed that *in vitro*, gangliosides through their sialic acid or phospholipids could play the role of receptors for adapted laboratory strains (Superti *et al.*, 1986; 1984b). In animals, nicotinic acetylcholine receptor (nAChR) has been suggested to be the rabies virus receptor (Lentz *et al.*, 1984; 1982) and an interaction between the α subunit of nAChR and purified rabies virus has been demonstrated in an overlay assay (Gastka *et al.*, 1996). However, direct evidence that this molecule is a receptor in animal is still lacking and recently, more conclusive experiments have indicated that neuronal cellular adhesion molecule (NCAM) and the low affinity nerve growth factor receptor (NGFR) can play the role of receptors for rabies virus in animals (Thoulouze *et al.*, 1998; Tuffereau *et al.*, 1998).

After binding, the virions enter the cell by the endocytic pathway. Subsequently, the viral envelope fuses with the endosomal membrane to allow the release of the viral genome into the cytoplasm. Fusion is triggered by the low pH of the endosomal compartment and is mediated by the viral glycoprotein.

The amino acid sequence of many rhabdoviral glycoproteins is known and many sequence alignment have been proposed. Rhabdoviral G proteins are type I membrane glycoproteins. The complete mature glycoprotein (after cleavage of the aminoterminal signal peptide) is about 500 amino acids long (505 for rabies virus, 495 for VSV Indiana). The large majority of the mass of the glycoprotein is located outside the viral membrane and constitutes the aminoterminal ectodomain. The ectodomain is followed by a single transmembrane hydrophobic segment which probably adopts an α -helical conformation. The rest of the protein is intraviral and probably interacts with internal proteins (N or M) (Mebatsion *et al.*, 1996).

For both VSV and rabies virus, it has been shown that G forms trimers (Gaudin *et al.*, 1992; Whitt *et al.*, 1991; Doms *et al.*, 1987; Balch *et al.*, 1986; Kreis and Lodish, 1986). This oligomeric organization is less stable than that of influenza HA and is sensitive to detergent solubilization (Gaudin *et al.*, 1992). In the case of VSV, there exists a dynamic equilibrium between monomers and trimers of G, both *in vitro* after detergent solubilization (Wilcox *et al.*, 1992; Lyles *et al.*, 1990) and *in vivo* in the endoplasmic reticulum (Zagouras *et al.*, 1991).

In electron microscopy, G appears to have a globular head above a stalk. Indications on the respective dimensions of the two part of the molecule have been obtained in the case of rabies virus. The length of the spikes extending from the viral membrane is about 8nm (Gaudin *et al.*, 1992).

Because the glycoprotein of rhabdovirus is the target of neutralizing antibodies, its antigenicity has been extensively studied. In the case of rabies virus, several hundred monoclonal antibodies (Mabs) have been used to characterize the antigenic structure of G making this protein one of the best known viral antigens available at the moment (Raux *et al.*, 1995; Benmansour *et al.*, 1991; Préhaud *et al.*, 1988; Seif *et al.*, 1985; Lafon *et al.*, 1983; Wiktor and Koprowski, 1980). RV G has two major antigenic sites which are antigenic site II and III. Antigenic site II is located between positions 34 and 42 and positions 198 and 200 (Préhaud *et al.*, 1988). These peptides are probably joined by a disulfide bridge and maintained together in the tertiary structure of G (Dietzschold *et al.*, 1982). Antigenic site III extends from amino acid 330 to 338 (Seif *et al.*, 1985). This site is associated with virulence and it has been shown that replacement of arginine 333 by any other amino acid (except lysine) leads to an avirulent phenotype (Coulon *et al.*, 1998; Tuffereau *et al.*, 1989; Seif *et al.*, 1985). Beside these major antigenic sites, one minor antigenic site and a few isolated epitopes have been described (Lafay *et al.*, 1996; Raux *et al.*, 1995; Benmansour *et al.*, 1991; Dietzschold *et al.*, 1990; Lafon *et al.*, 1983). The Mabs used in these studies turned out to be powerful tools to investigate the low pH-induced conformational changes of the glycoprotein which will be discussed below (Gaudin *et al.*, 1997; 1995b; 1993; 1991).

3.2. Fusion Properties of Rhabdoviruses

In the early 80s, cells infected with VSV and thus expressing VSV G at their surface were found to form syncytia when incubated at low pH (White *et al.*, 1981). Later, it was demonstrated that both VSV and RV were able to induce lysis of erythrocytes under acidic conditions (Gaudin *et al.*, 1991; Mifune *et al.*, 1982). In addition, ammonium chloride (a weak base which increases the pH of endosomes) was shown to inhibit rabies virus infection in cell culture (Superti *et al.*, 1984a). All these results were consistent with rhabdoviruses entering the cell by the endocytic pathway and subsequently fusing with the membrane of the endosome after its acidification.

Fusion of RV and VSV with artificial liposomes (Gaudin *et al.*, 1996; 1995b; 1993; 1991; Yamada and Ohnishi, 1986) and with cells (Puri *et al.*, 1992; 1988; Clague *et al.*, 1990) has been studied in detail. From these studies, it appears that neither RV (Gaudin *et al.*, 1991) nor VSV (Yamada and Ohnishi, 1990; Herrmann *et al.*, 1990) have a specific lipid requirement for fusion. The pH dependence of fusion is very similar from one rhabdovirus to another. For rabies virus, fusion is optimal around pH 6 and is still detected at pH 6.2–6.3 (Gaudin *et al.*, 1995b; 1993). In the case of VSV,

residual fusion activity is still observed at pH 6.5. More recently, we have identified a highly virulent field strain of viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus, for which fusion was optimal up to pH 6.6 and still observed at pH 6.9 (Gaudin *et al.*, 1999a). For this virus, there is a correlation between the pH at which fusion occurs and the magnitude of virulence: the lower is the pH-threshold for fusion, the more attenuated is the virus suggesting that the pH threshold for fusion could be a determinant for virulence. These results are consistent with results obtained on VSV that suggested that a shift toward lower values of the pH-threshold of fusion could lead to a loss of infectivity for cell culture (Frederiksen and Whitt, 1998; 1996).

When fusion kinetics was studied, it appeared that fusion of rhabdoviruses was preceded by a lag time, the duration of which increases with lower temperature and higher pH. In the case of VSV, this lag time is detected in experiments where virus is prebound to target cells (Clague *et al.*, 1990) and therefore is not due to slow binding of viral particles to target membranes. Most probably, as for other viral families, this lag time reflects the slow formation of structural intermediates implicating the membranes and a few viral glycoproteins at the fusion site and preceding membrane continuity.

As for influenza virus, preincubation of the rhabdoviruses at low pH in the absence of a target membrane leads to inhibition of the viral fusion properties (Gaudin *et al.*, 1991; Clague *et al.*, 1990). This has been particularly studied in the case of rabies virus (Gaudin *et al.*, 1996; 1995; 1993). For this virus, the kinetics of inactivation indicated that this inactivation phenomenon was more rapid when the temperature was high and the pH was low. There are two major differences between the low pH-induced inactivation of the fusion properties of rhabdoviruses and that of influenza virus. First, for rabies virus, the threshold pH for fusion (6.3) is lower than the pH threshold for inactivation (6.7) whereas fusion and fusion inactivation occur at the same pH for influenza virus. Second, fusion inactivation of rhabdoviruses is **reversible** after readjustment of the pH to above 7 whereas with influenza virus, inactivation is **irreversible**.

3.3. Low pH-Induced Conformational Changes of Rhabdovirus G

3.3.1. One Protein, Three Conformational States

As G is the only outer component of rhabdoviruses, it was obvious that it was directly involved in the fusion process. Indeed, when VSV G or RV G are transiently expressed in mammalian cells in the absence of the other viral proteins, they are able to induce syncytia formation at low pH (Whitt

et al., 1991; Florkiewicz and Rose, 1984; Riedel *et al.*, 1984). Furthermore, virosomes in which purified VSV G was reincorporated were found to have the same fusion properties as intact virus (Metsikkö *et al.*, 1986).

It was then logical to investigate putative low pH-induced structural transitions of G which would be responsible for activation and/or inactivation of the viral fusion properties. Low pH-induced conformational changes for a rhabdoviral glycoprotein were first demonstrated on a monomeric soluble form of VSV G which was cleaved from the virion with cathepsin D. This soluble form was shown to acquire hydrophobic properties when incubated at low pH (Crimmins *et al.*, 1983). Later, Doms *et al.* (1987) showed that VSV G solubilized with TRITON X100 and centrifuged through a sucrose gradient is monomeric at pH 7.4 and trimeric at pH 5.9. Therefore, the trimeric form of G is stabilized at low pH. Trimer stabilization and also acquisition of hydrophobic properties were reversible after restoring the pH to 7.

Low-pH induced structural changes of RV G have also been described on the basis of electron microscopy, sensitivity to proteases and Mab binding assays. Immediately after acidification below pH 6.7, the virions appear to be more hydrophobic (Durrer *et al.*, 1995; Gaudin *et al.*, 1993; 1991). This increased hydrophobicity results in viral aggregation (in the absence of a target membrane) (Gaudin *et al.*, 1991). It is also responsible for the hemagglutinating properties of the virus which are optimal at slightly acidic pH (about 6.4) (Gaudin *et al.*, 1991; Halonen *et al.*, 1974; Kuwert *et al.*, 1968) and reflect the ability of the virion to interact with a target membrane in a manner different from that at neutral pH. This hydrophobic interaction is mediated by G in an activated state (A) and probably constitutes a first step in the fusion process. However, a second step of protonation, lowering the pH below 6.3, is necessary for fusion to proceed. When viral aggregation is induced in the cold at pH 6.4 and 6.7, the shape of the spikes seems to be the same as at pH 7.4. Therefore, the transition from the native spikes (present at the viral surface at neutral pH) to the activated state (A) is not due to a large conformational change of RV G but more probably to a local change resulting in exposure of a previously buried hydrophobic region at its surface.

Prolonged incubation at low pH leads to a subsequent conformational change in G. In electron microscopy, the spikes then have a different morphology from that observed at neutral pH: in contrast to the well-defined 8nm-long native spikes, low pH-spikes form a rather wavy layer of 11nm wide (Figure 2). The appearance of these lengthened spikes is associated with the appearance of the low pH inhibition of viral fusion. This indicates that G in its low pH lengthened conformation is in an inactive state (I) unable to induce membrane fusion. Finally, from these results, it appears

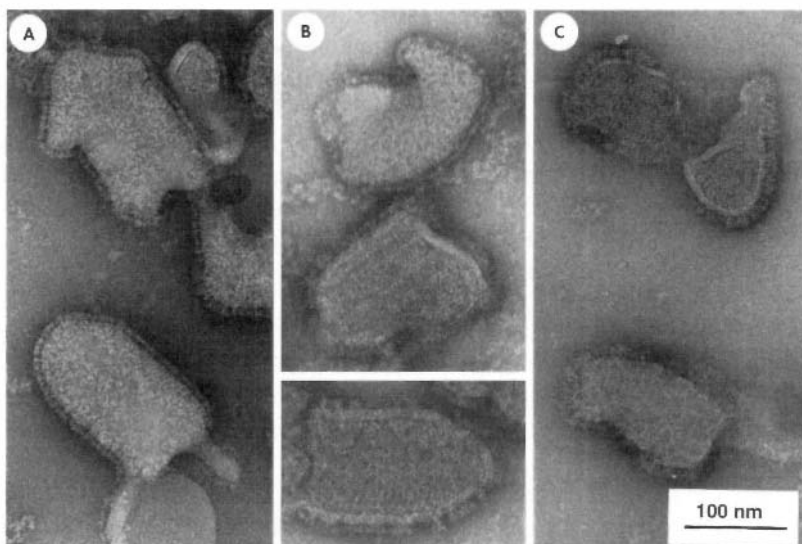


FIGURE 2. Electron micrographs of native and low-pH incubated rabies virus (CVS strain), negatively stained with 1 % sodium silicotungstate. All micrographs have the same magnification. (A) Native rabies virus with native, mushroom-shaped spikes (glycoprotein G). Note that some of the spikes in end-on view show characteristic triangular shapes. (B) Virus after 2 h of incubation at pH 6.7 and 37°C showing patches of native spikes alternated by patches of low-pH, lengthened spikes. (C) Virus after 2 h of incubation at pH 6.4 and 37°C. All particles show the lengthened spikes.

that RV G is able to adopt at least 3 conformations which are the native state (N) detected above pH 7, the activated state (A) which is responsible for viral aggregation and the inactive state (I) which appears to be lengthened in EM. These results are consistent with results obtained on VSV for which the existence of such states was postulated on the basis of an analysis of the kinetics of fusion (Puri *et al.*, 1992; 1988; Clague *et al.*, 1990).

Differently from other viral families, in the case of rhabdoviruses, low pH-induced conformational changes were **reversible** and when the virions which had been previously incubated at low pH were reincubated above pH 7, their spikes recovered their native aspect. Interestingly, even the low pH-induced insertion of the fusion domain of VSV in the target membrane has been demonstrated to be reversible (Pak *et al.*, 1997).

The sensitivity of RV G to proteolytic cleavage was also investigated (Gaudin *et al.*, 1995b; 1991). It was shown that G in its fusion inactive conformation is more sensitive to proteolysis than in its neutral pH structure.

In fact, cleavage of RV G in its fusion inactive conformation by any protease tested generates a fragment which approximately corresponds to the ectodomain of the glycoprotein (Gaudin *et al.*, 1991). This suggests that a region located in the ectodomain close to the transmembrane domain is more accessible to proteases in the fusion inactive conformation than in the native conformation. In contrast to these results, detergent solubilized VSV G protein becomes increasingly resistant to digestion with trypsin as the pH is lowered (Fredericksen and Whitt, 1996).

Finally, RV G conformational changes were also analyzed using a panel of monoclonal antibodies and the antigenicity of G in its native and its fusion inactive conformation was compared. It was shown that MAbs which recognize antigenic site II (aa 34 to 42 plus aa 198 to 200) at the surface of the native glycoprotein are unable to bind G in its fusion inactive conformation. Therefore, site II is disrupted during the structural transition from N to I. Major antigenic site III (aa 330 to 338) was also modified but to a lesser extent (Gaudin, Raux and Flamand, unpublished results). The accessibility of a linear epitope located between amino acids 255 and 270 was also shown to be restricted after the structural transition toward the I state (Gaudin, 1997) whereas minor antigenic site a (defined by antigenic mutants having mutations in position 342 and 343) does not seem to be modified.

The fact that the conformational changes were reversible indicated the existence of a pH-dependent equilibrium between the different states. Using monoclonal antibodies directed against antigenic site II which, as mentioned above, bound only to the native state, the fraction of the glycoprotein in the fusion-inactive conformation at different pH values could be calculated. When the equilibrium was reached at 37°C, this fraction was about 50% at pH 6.7, about 80% at pH 6.4 and more than 95% below pH 6. All these results were in remarkable agreement with the estimates obtained from EM micrographs (Gaudin *et al.*, 1993).

3.3.2. Identification of the Fusion Domain of Rhabdoviruses

Unlike influenza hemagglutinin (HA), rhabdovirus glycoproteins do not undergo an activating cleavage to generate an amino-terminal fusion peptide. Furthermore, primary sequence analysis of rhabdovirus glycoprotein neither reveals any hydrophobic sequences (except the signal sequence and the transmembrane domain) nor obvious homologies with other fusion peptides. However, for VSV, it was suggested that a conserved uncharged sequence (aa 118–136, see Figure 3) which, however, contains polar amino acids could play the role of a fusogenic domain (Ohnishi, 1988). Indeed, linker insertion or single mutations in VSV G (see Table 1) in or near this

117 QGTWLNPGFPPQSCGYATVT 136

FIGURE 3. Sequence of the putative fusogenic peptide of VSV (Ohnishi, 1988).

Table 1
Mutations in the putative fusogeuk peptide of VSV and their effect on membrane fusion. The mutant glycoproteins were expressed in cells and assayed for low pH-induced syncytia formation. ¹ from Fredericksen and Whitt (1995), ² from Zhang and Ghosh (1994) and ³ from Fredericksen and Whitt (1996)

T119A ¹	fuses as wild type
T119D ¹	fuses as wild type
L121D ¹	fuses as wild type
L121K ¹	fuses as wild type
P123A ¹	fuses as wild type
P123G ¹	fuses as wild type
P123L ²	fuses as wild type
G124A ²	fusion poorly efficient detected only below pH 6
G124E ¹	fusion detected only below pH 5.5
F125A ¹	no cell surface expression of G
F125D ¹	no cell surface expression of G
F125Y ²	fusion poorly efficient
P127D ¹	fusion poorly efficient detected only below pH 5.5
P127G ²	fusion poorly efficient detected only below pH 6
P127L ²	fusion poorly efficient detected only below pH 6
Q128R ¹	fuses as wild type
G131A ¹	fuses as wild type
A133K ¹	no fusion activity
D137L ^{1,3}	fusion slightly less efficient than wt VSV
D137N ²	fusion poorly efficient detected only below pH 5.5
E139I ¹	fuses as wild type
E139L ^{1,3}	fusion slightly less efficient than wt VSV
E139R ¹	no cell surface expression of G
E139S ¹	fuses as wild type
E139T ¹	fuses as wild type

region affect the fusion properties of the glycoprotein (Fredericksen and Whitt, 1996; 1995; Zhang *et al.*, 1994; Li *et al.*, 1993).

To investigate direct interactions between the glycoprotein and the membrane, hydrophobic photolabelling, a technique based on the ability of photoactivatable lipids to covalently modify the domains of proteins which interact with the inner hydrophobic core of the membrane, was used for both VSV and RV (Durrer *et al.*, 1995). This technique has already been used with success to study the interaction between influenza HA and

membranes during the fusion process (Weber *et al.*, 1994; Tsurudome *et al.*, 1992; Stegmann *et al.*, 1991) and has been described in other reviews (Gaudin *et al.*, 1995a; Brunner, 1993; 1989). Briefly, the reagents used for such studies are very hydrophobic molecules often structurally related to phospholipids. Their localization is therefore restricted to the hydrophobic membrane phase. When irradiated by UV, these reagents generate *in situ* (i.e. in the inner core of the membrane) highly reactive and very short-lived groups such as carbene. The photoreagent used to study the interactions between the rhabdovirus glycoproteins and target membranes was [¹²⁵I]TID-PC/16 (Durrer *et al.*, 1995).

Large unilamellar vesicles (LUVs) containing [¹²⁵I]TID-PC/16 were incubated with RV and VSV under various conditions before irradiation (Durrer *et al.*, 1995). The results were similar for RV and VSV. Radioactivity was only found associated with G. This labeling was pH dependent: the amount of radioactivity bound to G was about 70 times more when irradiation was performed after a one minute-incubation at pH 6 and 23°C than after a one-minute incubation at pH 7 and 23°C. A significant increase of labelling of G was also observed when the virions were incubated with the radioactive LUVs at pH 6.4 and 0°C. This confirmed that under these conditions of pH and temperature, although no fusion is detected, G is in an activated state which is already able to interact with the target membrane in a hydrophobic manner.

After UV photolysis, the purified glycoprotein (electroeluted from SDS-PAGE) was submitted to CnBr fragmentation to generate peptides of suitable size which were then analysed by SDS PAGE. For both RV G and VSV G, two domains of the glycoprotein were found to contain radioactivity. As expected, after membrane fusion at pH 6 and 23°C, radioactivity was found in peptides corresponding to the transmembrane domain. Radioactivity was also found in peptide [102–1791 of RV G and [58–2211 of VSV G which contained the putative fusion domain of VSV G defined on the basis of the previously described mutagenesis experiments. Prefusion conditions (pH 6.4 and 0°C) resulted in preferential or exclusive labeling of these peptides. Therefore, as for influenza virus (Tsurudome *et al.*, 1992; Stegmann *et al.*, 1991), a peptide of the rhabdovirus G ectodomain interacts with the target membrane before membrane continuity.

The uncharged peptide of VSV defined by Ohnishi (1988) contains a cysteine in position 130. This cysteine is conserved among vesiculoviruses but is not found in lyssaviruses sequence (Durrer *et al.*, 1995). In the case of viral hemorrhagic septicemia virus (VHSV), this cysteine is in position 152 and has been demonstrated to form a disulfide bridge with cysteine 110 (Einer-Jensen *et al.*, 1998). Interestingly, the only natural mutation which has been shown to affect the fusion pH of VHSV is not located in the region

from aa 144 to 154 which is homologous to the fusion peptide of VSV as defined on the basis of mutagenesis experiments but in position 118 (Gaudin *et al.*, 1999a). Glutamine 118 is separated from cysteine 110 by the heptapeptide STSFFGG. As phenylalanine and glycines are often found in viral fusion peptides, this peptide may also interact with one membrane during the fusion process. It is thus reasonable to think that the complete fusion domain of VHSV is constituted by two distant regions of the primary sequence put together in the tertiary structure of the glycoprotein by disulfide bridge between cysteines 110 and 152.

3.3.3. Mutations Affecting G Conformational Changes

When the location of the VSV fusion peptide was investigated by mutagenesis, it appeared that linker insertion in other domains of the protein than the putative fusion peptide also affects the fusion properties of the glycoprotein (Li *et al.*, 1993). In particular, linker insertion before aa 390 and 410 resulted in the loss of the fusion properties of VSV G. Mutations located in the same domain of the molecule were also shown to affect its properties (Shokralla *et al.*, 1998). As this region of G does not seem to interact with the target membrane, it has been proposed that mutations in this domain somehow affect the low pH-induced conformational changes of G.

In the case of rabies virus, monoclonal antibodies which recognize only the fusion inactive (I) conformation of RV G on the viral surface have recently been described (Raux *et al.*, 1995). These Mabs were neutralizing when they were incubated with rabies virus which had been previously incubated at pH 6.4 and 37°C for 2h in order to induce the conformational change toward the fusion inactive conformation of G. It was thus possible to select mutants escaping neutralization (Raux *et al.*, 1995). These mutants were called RAIN mutants for resistant to acid-induced neutralization. The mutants could be classified in two groups (Gaudin *et al.*, 1996). The first group contains mutants with mutations in position 10, 13 and 15. These mutations were shown to be located in the epitope recognized by the Mabs and thus decrease the affinity of the Mabs for the virus. The mutations of the mutants of the second group were rather dispersed along the primary sequence (mutations in amino acids 44, 282, 392 and 396). These mutants, although having the same fusion properties as the parental strain, were affected in the kinetics of their conformational change toward the I state. In particular, for mutants in position 44, 392 and 396, the kinetics of the conformational change toward the I state were considerably slowed down. Therefore, not enough spikes were in the fusion inactive conformation after 2 h at pH 6.4 to allow the binding of a sufficient number of antibodies to

neutralize the virus. As the structure of RV G is not known, it is difficult to propose a role for methionine 44, valine 392 or methionine 396 in the low pH structural transition. However, amino acids 392 and 396 are located near histidine 397 and proline 398 which are conserved among all rhabdovirus glycoproteins. This motif is located between two putative alpha helices predicted more or less strongly for all rhabdoviruses. Interestingly, the linker insertions in VSV G (Li *et al.*, 1993) and the mutants of Shokralla *et al.* (1998) that affect the fusion properties of G are located in the first putative helix. Thus, this domain of the rhabdovirus glycoprotein seems to play a role in the control of the conformational changes and it is possible that the conserved His-Pro motif act as a hinge during the structural transitions of G.

Finally, in the case of VHSV, genetic evidence has been obtained that indicates that this domain controlling G conformational changes is structurally linked to an antigenic loop located between aa 130 and 143 just before the carboxy terminal part of the fusion domain (Gaudin *et al.*, 1999a). Whether these two interacting domains form a structure similar to the one recently determined for the fusion protein of retroviruses (Weissenhorn *et al.*, 1997; Chan *et al.*, 1997; Fass *et al.*, 1996; Lu *et al.*, 1995) remains to be determined.

The influence of the transmembrane (TM) domain sequence of rhabdovirus glycoprotein on fusion and low pH-induced conformational changes has also been investigated. In the case of VSV, the first results suggested that membrane anchoring by a hydrophobic TM peptide (but not a glycoposphatidylinositol anchor) was necessary but that the specific aa sequence of this TM domain had no influence on fusion (Odell *et al.*, 1997). However, other results indicated that mutations of two glycines of VSV G TM domain affected the glycoprotein fusion properties (Cleverley and Lenard, 1998). For RV, we have demonstrated that in the absence of the TM domain, the soluble ectodomain folds in a monomeric conformation which has the antigenic characteristics of the fusion inactive conformation whatever the pH (Gaudin *et al.*, 1999b). Thus, in this case, the inactive state is the more stable state and the native state is never detected.

3.3.4. Role of the Fusion Inactive State

As mentioned in the paragraph devoted to influenza, although the major structural transition of HA leads to fusion inactivation in the absence of a target membrane, it plays a decisive role in the fusion process. The situation in rhabdoviruses appears to be rather different. First, as mentioned above, the conformational changes are **reversible** and there is a pH dependent equilibrium between the different forms of G. Second, the fusion

inactive state is clearly present at a pH at which fusion is not detected (pH 6.7 in the case of RV). Finally, although their structural transition toward the fusion inactive state is affected, the RAIN mutants have the same fusion properties as the parental strain. From these observations, it has been concluded (at least for RV) that the transition toward the I state is irrelevant to the fusion process.

What could then be the role of the fusion inactive state? An answer to this question came when the conformation of G during its transport through the Golgi apparatus was studied. In immunofluorescence, it appeared that intracellular G was not recognized by Mabs specific of the native conformation whereas antibodies able to recognize the fusion inactive conformation efficiently recognized intracellular G in the Golgi apparatus. Furthermore, when infected cells were treated by monensin before fixation and permeabilization, antibodies specific for the native conformation were then able to bind G in intracellular compartments. The most simple explanation was that G was transported through the Golgi in a non-native conformation antigenically similar to the fusion inactive state. Monensin, by equilibrating the pH of the transport vesicles which are known to be acidic (Anderson and Orci, 1988), allowed the structural transition toward the N state. Thus, a plausible role for the fusion inactive state is to avoid undesirable fusion in the acidic Golgi vesicles during G transport.

In fact, it appears that each viral family for which fusion is triggered by low pH has developed the ability to avoid undesirable fusion during the transport of the fusogenic glycoprotein through the acidic compartment of the Golgi apparatus. For example, in influenza virus, the pH of the transport vesicle is regulated by the ion channel M2 protein (Sugrue *et al.*, 1990). Furthermore, for this virus, the activating cleavage of HA0 into HA1 and HA2, which is necessary to generate the aminoterminal fusion peptide of HA2, occurs at a late step during transport. In the case of Semliki forest virus, the spikes are transported as a trimer of a heterodimer p62E1 precursor. Cleavage of p62 into E2 and E3 is necessary for E1 to promote viral fusion under biological conditions. This cleavage also occurs at a late step of transport shortly before viral budding (Lobigs and Garoff, 1990). In a very similar manner, for tick-borne encephalitis virus, the fusogenic glycoprotein E is transported in association with the prM protein which protects E from undergoing an irreversible conformational change in the Golgi acidic compartments. Cleavage of prM into M protein at a late stage of transport allows E to become fusogenic. In the case of rhabdoviruses, the glycoprotein would be transported in a fusion inactive conformation and the reversibility of the low pH-induced inactivation process would be necessary to allow the incorporation of G in its native state in newly formed virions.

3.3.5. Other Differences between Rhabdoviral G and Influenza Virus HA Conformational Changes

Although the reversibility of the low pH-induced conformational changes of rhabdoviral glycoproteins is the most obvious difference with other fusogenic glycoproteins, there are other differences that exist between rhabdovirus G and influenza HA and have to be underlined.

First, as mentioned in paragraph 2.2, recent elucidations of the structure of the protease resistant core of HA (TBHA2) (Bullough *et al.*, 1994), of the TM fusion protein of retroviruses (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997; Fass *et al.*, 1996) and of a recombinant fragment of gp2 of Ebola virus (Weissenhorn *et al.*, 1998) but also structural datas obtained on the F protein of paramyxoviruses (Bagai Joshi *et al.*, 1998) have shown intriguing structural similarities. Together with the atomic structure of a recombinant synaptic fusion complex (Sutton *et al.*, 1998), these structures have suggested that the formation of coiled-coils could be a general feature during activation of fusogenic glycoproteins. It is noteworthy that there is no coiled coil predicted for rhabdovirus glycoproteins and most probably rhabdoviral fusion does not involve the formation of such structures.

Second, in all the structure of viral fusogenic proteins determined up to now, it appears that the transmembrane domain and the fusion peptide of the fusogenic proteins are colocated at the same extremity of the coiled-coils (Skehel and Wiley, 1998). As these structures are supposed to correspond to the conformation of the protein at the end of the fusion process, this feature reflects the fact that, after fusion, the TM domain and the fusion peptide of the protein are located in the same fused membrane. As a consequence, for these proteins, when the conformational change is triggered in absence of a target membrane, the fusion peptide penetrates the viral membrane. This has been demonstrated nicely in the case of influenza HA2 using a hydrophobic radioactive photoactivatable phospholipid analogue that was incorporated in the viral membrane (Weber *et al.*, 1994). In the case of VSV and RV, unlike influenza, photolabeling experiments have suggested that no fusion-independent pathway for viral membrane insertion exists (Durrer *et al.*, 1995).

Third, when the structures of membrane inserted aminoterminal fusion peptides were studied by infrared spectroscopy, it appeared that they all adopted an alpha helical conformation and their ability to induce membrane fusion has been related to this structural characteristic (Durell *et al.*, 1997; Martin *et al.*, 1996; Luneberg *et al.*, 1995). However, the fusion peptide or rather the fusion domain of rhabdovirus G is internal and it is possible that it adopts a conformation different from aminoterminal fusion peptides upon membrane insertion.

4. ATTEMPT TO RECONCILE THE DATA OBTAINED ON RHABDOVIRUSES WITH THOSE OBTAINED ON OTHER VIRAL FAMILIES

4.1. Existence of Reversible Steps in Fusogenic Glycoproteins Conformational Changes

As mentioned in the introduction, existing data on an increasing number of viruses suggest that metastability of the native state and irreversibility of the fusogenic conformational change is a common feature of viral fusion glycoproteins regardless how the conformational change is triggered and it has been proposed that the energetically expensive membrane fusion reaction could be driven by the energetically favorable conformational change (Carr *et al.*, 1997). However, although the native state of these fusogenic glycoproteins is metastable, the complete conformational change is a multistep process of which the earliest steps have often been shown to be reversible.

When HA conformational changes are monitored by tryptophan fluorescence spectroscopy, after exposure to pH 4.9, reversibility is detected at short times but is progressively lost after prolonged low-pH incubation (Krumbiegel *et al.*, 1994). Similar results have been obtained when HA structural transitions were studied by near UV CD (Korte *et al.*, 1997) or bis-ANS binding studies (Korte *et al.*, 1994). Here again, the extent of the reversibility declined with the duration of low pH exposure.

In the case of SFV, the dissociation of the E1-E2 dimer upon acidification which is the first step of the low pH-induced conformational change has been shown to be reversible (Wahlberg *et al.*, 1989). Similar conclusions have been drawn on TBEV E glycoprotein for which the dissociation of dimeric E into monomer which precedes the irreversible reoligomerization into trimers is also reversible. For this glycoprotein, it is noteworthy that the soluble proteolytically cleaved sE dimer is able to dissociate at low pH but fails to reassociate into trimers. In the absence of the transmembrane domain of E, the low pH-induced conformational change is reversible and the protein reassociates into dimers upon restoring the pH to neutral (Stiasny *et al.*, 1996).

Thus, partial reversibility of conformational changes related to fusion may be common but complete reversibility remains a characteristic of the rhabdovirus family.

4.2. How do Rhabdoviruses Overcome the High Energetic Barrier Encountered During Fusion?

As rhabdoviruses are confronted to the same energy barrier to induce membrane fusion, they probably form the same membranar intermediates

as those encountered in other fusogenic systems because this pathway is certainly optimal from an energetical point of view. Indeed, the use of inverted cone shaped lipids (such as lysophosphatidylcholine) and cone shaped lipids (such as oleic acid) to investigate the membrane fusion intermediates suggest that the structure of these intermediates are similar to those postulated in other systems (Gaudin, unpublished results).

The fact that rhabdovirus conformational changes are reversible suggests that the quantity of energy released during the structural transition is not as large as in other viral fusogenic glycoproteins for which the fusogenic transition is irreversible. One way to overcome this problem would be to make a bigger fusion complex made of a larger number of fusogenic glycoproteins acting in a concerted manner. However, in the case of VSV, Bundo-Morita *et al.* (1988) have shown that the size of the fusogenic complex was about 5 trimers. Interestingly, some of the RV RAIN mutants affected in the kinetics of their low pH-induced structural transition showed a hexagonal lattice of G at their surface when briefly incubated under pre-fusion conditions (pH 6.6 and 0°C) (Gaudin *et al.*, 1996). It is possible that one hexagon (made up of 6G trimers) constitutes a minimal prefusion complex. Therefore, the number of rhabdoviral spikes making up the fusion complex would be the same as that for other viral systems (Danieli *et al.*, 1996; Blumenthal *et al.*, 1996; Plonsky and Zimmerberg, 1996).

There are two other ways for rhabdoviral G to overcome the energetic barrier and to drive the fusion reaction (Figure 4). First, as mentioned in part 3.3.4, whether the transition towards the fusion inactive conformation plays a role during the fusion process is unclear and it has been suggested that the structural transition toward the inactive state is irrelevant to the fusion process (Gaudin *et al.*, 1995b). Therefore, as the presence of the activated state is not sufficient to induce fusion, it remains possible that another conformational change that has not been identified exists, which would occur only when G is anchored into the target membrane by its fusion peptide. This putative unidentified fusogenic structural transition triggered by the interaction between the fusion domain and the target membrane could be also irreversible for rhabdoviruses and, thus, could release enough energy to drive the fusogenic reaction (Figure 4A). Second, at the end of part 3.3.1, we have mentioned that there is a pH dependent equilibrium between the different conformations of rabies virus glycoprotein. Above pH 7, an antibody which recognized only the fusion inactive conformation at the viral surface is able to bind the virion provided the incubation with the virus is long enough (Raux *et al.*, 1995). Thus, this antibody is able to shift the equilibrium toward the fusion inactive state by sequestering G in this conformation. This indicates that the transition from the native toward the fusion inactive state exists above pH 7. Between pH 6.4 and 6.7, after long incubations at 37°C, both the fusion inactive and the native

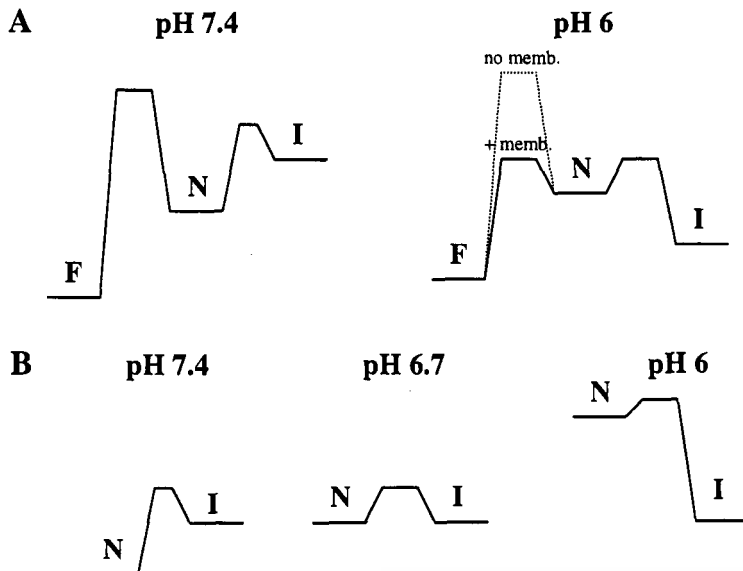


FIGURE 4. Possible free energy diagrams for the different conformations of G at different pH values. A) The transition from the native state (N) to the fusion inactive state (I) is irrelevant to the fusion process. The fusogenic transition is under kinetic control and is possible only at acidic pH when G interacts with the target membrane by its fusion domain. In the absence of a target membrane, at acidic pH, G is in its fusion inactive conformation. When G is not in the F state (which is the result of the fusogenic transition), it could reverse to its native state by raising back the pH above 7. B) The transition from the native state (N) to the fusion inactive state (I) is the fusogenic transition but releases enough energy to drive the fusion reaction only below pH 6.3. Although N is metastable at pH 6, the structural transition is reversible by raising the pH back above 7.

conformation are detected (Gaudin *et al.*, 1993). Thus, the equilibrium between the different conformations of G is clearly detected above pH 6.4. However, below pH 6.4, most of the spikes if not all are in the fusion inactive state and there is no evidence that at these pHs the transition from the fusion inactive state toward the native state is possible. In other words, it is possible that although the structural transition is reversible by raising the pH back above 7, **the native state is metastable below pH 6.4** and thus, at fusogenic pH, the transition from the native (or the activated conformation) toward the fusion inactive state releases enough energy to drive the fusion reaction (Figure 4B).

4.3. Final Remarks

Although reversibility of the low pH-induced conformational change is a unique feature of rhabdoviruses among the enveloped viruses, it should be noted that reversibility of SNARE complexes implicated in cellular vesicle fusion has been demonstrated. The disassembly of these fusion complexes is catalyzed by the N-ethylmaleimide sensitive factor (NSF) ATPase and is an important component of the recycling process necessary for new vesicles formation (Nicholson *et al.*, 1998; Hanson *et al.*, 1997).

In the case of viruses, the reversibility is not necessary: viral fusion proteins are used only once. Premature activation is prevented by receptors downregulation in the case of retroviruses and by all the means described in part 3.3.4 for viruses fusing at low pH. This is not the case for rhabdoviruses and most probably, G undergoes a conformational change in the acidic Golgi compartment. The reversibility is thus necessary for G being incorporated in a native functional conformation in neosynthetized virions.

ACKNOWLEDGMENTS. I thank Rob Ruigrok and Marcel Knossow for discussions and critical comments on the text. This work is supported by CNRS (UPR 9053).

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Specific Roles for Lipids in Virus Fusion and Exit

Examples from the Alphaviruses

Margaret Kielian*, Prodyot K. Chatterjee, Don L. Gibbons, and Yanping E. Lu

1. INTRODUCTION

Enveloped viruses infect cells by fusion of the viral membrane with a cellular membrane. Following replication, progeny virus particles are generally formed by budding through a cellular membrane, thus acquiring both the viral spike protein(s) and a lipid bilayer. The goals of current studies of virus membrane fusion and budding are two-fold: to use molecular information about these processes to develop specific and powerful anti-viral therapies, and to apply knowledge from the more accessible viral systems to help understand the identities and functions of proteins mediating cellular membrane fusion and budding processes. Alphaviruses such as Semliki Forest virus (SFV) have been particularly important for studies of membrane fusion. A key feature of the fusion reaction of this simple enveloped

MARGARET KIELIAN, PRODYOT K. CHATTERJEE, DON L. GIBBONS, and YANPING E. LU Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461. *Corresponding author.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

virus is that it requires the presence of cholesterol and sphingolipid in the target membrane. Cholesterol has also been shown to be required for the efficient exit of SFV from infected cells. To date, SFV is the first and best understood example of a virus with specific lipid requirements for fusion and exit. The mechanisms and wider implications of these lipid requirements are the central focus of this review.

In order to understand the interesting and important roles that cholesterol and sphingolipids play in the alphavirus lifecycle, the general features of alphavirus structure, replication, and assembly will be summarized, and our current molecular understanding of alphavirus membrane fusion will be discussed in some detail. The major emphasis of our review, however, is to describe what is known about the specific functions of cholesterol and sphingolipid in alphavirus infection, and their possible relevance to other virus and pathogen systems, rather than to comprehensively review the extensive body of research on alphaviruses. In each section, therefore, the reader will be referred to a number of excellent reviews for more in depth coverage of the areas that we do not describe in detail. The most extensive work on lipid requirements has used SFV as an experimental system, but more recent studies have characterized the alphavirus Sindbis virus (SIN) as well. We will review and compare results from the SFV and SIN systems as much as possible, which should help to highlight the key features of the lipid requirements that are likely to be general properties of the alphaviruses.

Alphaviruses have been critical in first demonstrating that particular lipids can play a key role in the virus lifecycle. At the end of this review, we will consider the available data suggesting a specific role of lipids in the entry and exit of other viruses and pathogens. While the evidence is as yet quite incomplete, there are already indications of the possible importance of lipids in a variety of systems. Hopefully the techniques, approaches, and information gained from the alphaviruses will help to point the way towards a better understanding of how lipids may act as important players in the membrane interactions of viruses and pathogens.

2. THE ALPHAVIRUS LIFECYCLE

Alphaviruses are a genus of the family *Togaviridae* containing about 26 viruses (see (Strauss and Strauss, 1994) for review). These viruses all have a lipid envelope and an RNA genome of positive polarity, and are transmitted in nature by arthropod vectors, primarily mosquitoes. Alphaviruses can infect a large range of host organisms including insects, small mammals, birds, and humans, and also readily infect tissue culture cells from a wide

variety of different hosts and tissues (Strauss and Strauss, 1994; Kielian, 1995). Some alphaviruses such as eastern equine encephalitis virus (EEE virus) and western equine encephalitis virus (WEE virus) can cause fatal encephalitis in humans and horses, while other alphaviruses such as Ross River virus can cause polyarthritis, fever, and rashes (Griffin, 1986). Both EEE virus and WEE virus are endemic to the United States and are responsible for periodic epidemics of encephalitis in humans (Anonymous., 1994; Anonymous., 1992). The alphaviruses SFV and SIN have low pathogenicity in humans, and have been widely used as experimental systems to study virus replication, assembly, the infectious entry pathway, and membrane fusion (Strauss and Strauss, 1994; Kielian, 1995). Such studies, particularly of viral entry and membrane fusion, take advantage of the fact that alphavirus growth, radiolabeling, and purification are well-characterized and extremely efficient, and that the virus as isolated has a high plaque forming unit/particle ratio (Kielian, 1995). These properties have made detailed biochemical analysis of alphavirus fusion both practical and meaningful. In addition, complete cDNA clones of SIN (Rice *et al.*, 1987), SFV (Liljeström *et al.*, 1991), and several other alphaviruses have made it possible to manipulate the virus sequence, transcribe infectious viral RNA *in vitro*, and introduce it into cells to generate virus infection. The highly symmetrical organization of alphavirus proteins, described below, has enabled the structural characterization of alphaviruses using cryo-electron microscopy (Strauss and Strauss, 1994; Cheng *et al.*, 1995; Fuller *et al.*, 1995). Thus the overall experimental potential and advantages of alphaviruses are high.

2.1. Virus Structure and Assembly

Alphaviruses are spherical viruses with a diameter of about 640–690 Å (Strauss and Strauss, 1994; Kielian, 1995; Fuller *et al.*, 1995; Cheng *et al.*, 1995; Paredes *et al.*, 1998). They contain one copy of the viral genome, a positive-stranded RNA of ~11.4kb that encodes the four subunits of the RNA replication complex and the structural proteins of the virus. The RNA is assembled with 240 copies of the capsid protein (~30kDa) to form the virus nucleocapsid, an icosahedrally symmetrical structure with triangulation number $T = 4$. The individual capsid proteins are arranged as pentamers and hexamers to form the spherical nucleocapsid.

The nucleocapsid is surrounded by a lipid bilayer derived from the host cell plasma membrane during virus budding. The lipid composition of the bilayer in general appears to reflect that of the host cell plasma membrane, and contains a cholesterol: phospholipid ratio of ~1 : 1 when the virus is

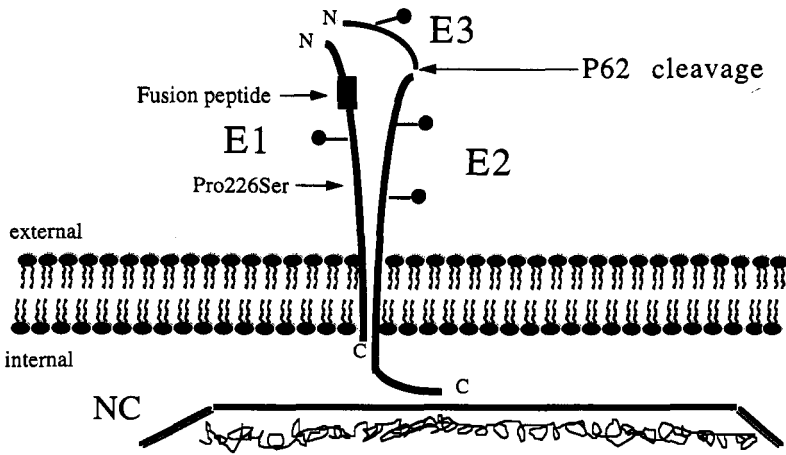


FIGURE 1. Schematic representation of the alphavirus spike glycoprotein and its interaction with the virus nucleocapsid. The ball and stick structures represent carbohydrate side chains. The location of the putative E1 fusion peptide, the p62 cleavage site, and the mutation (P226S) that affects *srf-3* cholesterol dependence are indicated. Figure is not to scale.

propagated in mammalian host cells such as BHK cells (Kielian, 1995; Strauss and Strauss, 1994).

The virus spike protein contains two type I transmembrane polypeptides, E1 and E2, which are each about 50 kDa and associate as a tight but non-covalent heterodimer (Figure 1). A peripheral spike polypeptide, E3, of ~10kDa is present in SFV particles but is released from the SIN spike protein and not associated with mature SIN particles. 240 copies of these spike polypeptides are present in each virus particle in a 1 : 1 ratio with the capsid protein, and are arranged in a $T = 4$ icosahedral protein shell. The virus spike protein structure is a trimer, $(E2-E1)_3$ in SIN or $(E3-E2-E1)_3$ in SFV, thus forming 80 spike proteins. The distal, tripartite projecting domain of the spike protein is triangularly-shaped and formed of the three E2-E1 pairs. More proximal to the membrane, this trimer separates to individual E2-E1 pairs. This separation forms a gap or cavity between the three spike stalks, which then traverse the membrane via the transmembrane domains of E1 and E2. Within the interior of the virus particle, each individual E2-E1 dimer interacts with a capsid monomer via the E2 internal domain. On the external surface of the virus, each trimeric spike also contacts adjacent spike proteins through lateral interactions to form a layer or "skirt" of spike protein that spreads over and covers most of the surface of the virus particle (Fuller *et al.*, 1995; Cheng *et al.*, 1995).

The capsid and spike polypeptides are synthesized as a polyprotein from a subgenomic RNA, in the order capsid-p62-6K-E1, and are post-translationally processed to produce the individual polypeptides (Strauss and Strauss, 1994; Kielian, 1995; Garoff *et al.*, 1994; Schlesinger and Schlesinger, 1986). p62 (termed PE2 in SIN) is a precursor to the mature E3 and E2 subunits. 6K is a small hydrophobic peptide that provides the signal sequence for E1 and is found at low levels in the mature virus particle. During virus infection, capsid protein is synthesized on free ribosomes, cleaves itself autoproteolytically from the nascent polypeptide chain, and assembles with viral RNA to form new nucleocapsids (Figure 2). A signal sequence on p62 then initiates translocation of the remainder of the polyprotein into the lumen of the rough endoplasmic reticulum (RER). The E1 and p62 subunits associate within the RER to form non-covalent heterodimers, and are transported as a complex through the RER and Golgi to the plasma membrane. Evidence suggests that this dimerization is required for proper spike protein folding and transport. Similar to cellular membrane proteins, E1 and p62 are glycosylated and fatty acylated during their transport through the RER and Golgi complex. The p62 precursor is cleaved after an ArgHisArg sequence to give E3 and E2. Cleavage probably occurs in or after the trans-Golgi network by the cellular protease furin (deCurtis and Simons, 1988; Strauss and Strauss, 1994). Following arrival at the plasma membrane, the spike proteins interact with the viral nucleocapsid via the E2 cytoplasmic domain to mediate the budding and release of progeny virus particles.

2.2. Virus Entry and Fusion

2.2.1. Endocytic Entry and Low pH-Triggered Fusion

SFV was the first virus demonstrated to infect cells via endocytic uptake and low pH-triggered fusion (Helenius *et al.*, 1980) (Figure 2), an overall pathway now known to be used by members of a number of virus families including orthomyxoviruses (Bentz, 1993; White, 1992; Wiley and Skehel, 1987), rhabdoviruses (Lenard, 1993), flaviviruses (Allison *et al.*, 1995), bunyaviruses (Gonzalez-Scarano, 1984), and adenoviruses (Greber *et al.*, 1993). The first step in alphavirus entry is the binding of virus to receptors on the plasma membrane. Receptor binding appears to occur primarily through the E2 subunit (Kielian, 1995; Strauss and Strauss, 1994), although there may be some involvement of the E1 subunit as well. Several molecules have been proposed as candidates for alphavirus receptors, including proteins such as the high affinity laminin receptor and class I major histocompatibility antigens (Wang *et al.*, 1992; Ubol and Griffin, 1991; Helenius

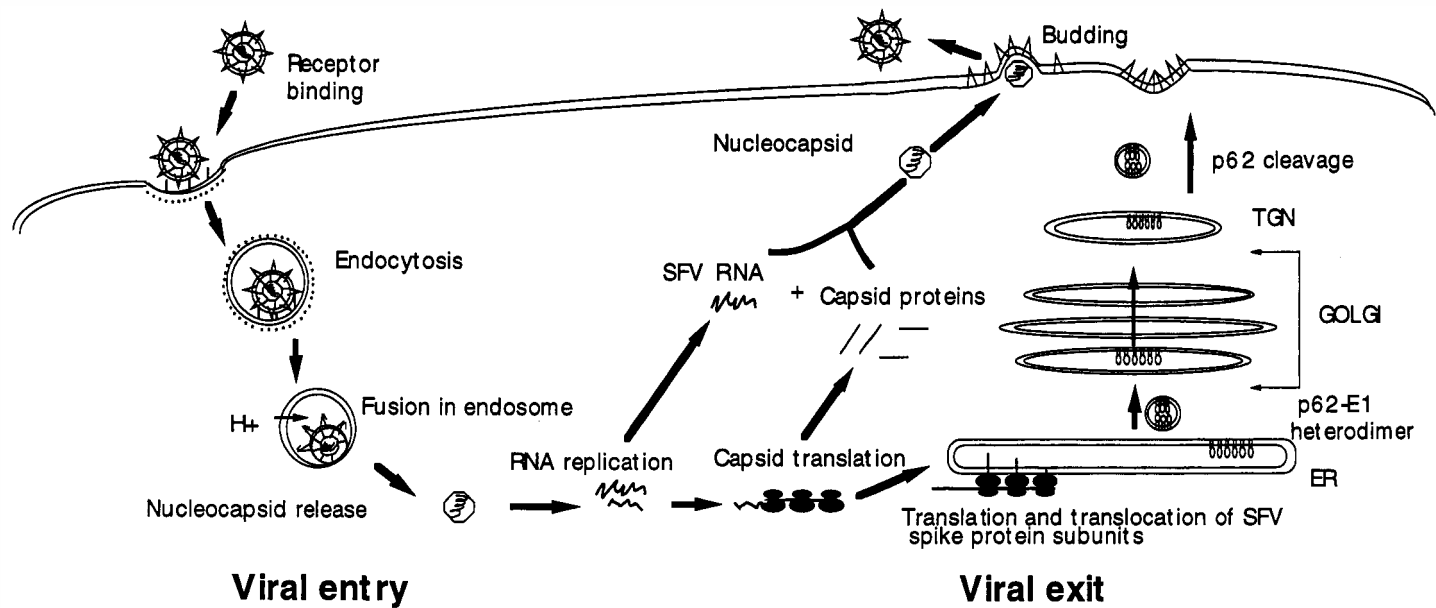


FIGURE 2. The alphavirus lifecycle. Figure taken from (Kielian, 1995) with permission.

et al., 1978), and more recently, cell surface heparan sulfate (Brynes and Griffin, 1998; Klimstra *et al.*, 1998). The available data suggest that a number of different molecules can act as alphavirus receptors, and this broad receptor usage is perhaps to be expected from the fact that alphaviruses infect such a range of different cell types and hosts.

Following receptor binding, the virus is internalized by receptor-mediated endocytosis, a constitutive pathway used by the cell to take up a variety of nutrients, growth factors, and hormones, and to control membrane homeostasis (Mellman *et al.*, 1986; Goldstein *et al.*, 1985) (see virus entry, Figure 2). Endocytic uptake of SFV has been demonstrated by a number of biochemical and morphological experiments (Helenius *et al.*, 1980; Kielian, 1995; Kielian, 1993). Uptake is rapid (half-time of ~10min in BHK cells or ~3–5min in CHO cells) and is inhibited by low temperature (such as incubation on ice). Electron microscopy has demonstrated that SFV endocytosis occurs via typical clathrin-coated endocytic vesicles (Helenius *et al.*, 1980), and delivery of anti-clathrin antibodies into the cytoplasm was shown to inhibit the ability of cells to internalize SFV and become infected (Doxsey *et al.*, 1987). Recently, the role of endocytosis in alphavirus infection was studied by using a dominant-negative dynamin mutant to block endocytic uptake (DeTulleo and Kirchhausen, 1998). Cells expressing this dynamin mutant were shown to be resistant to infection by both SIN and SFV.

Following endocytic uptake, the virus is transported to prelysosomal endosomes. The luminal pH of endosomes ranges from ~6.5 to 5.0 due to a proton-translocating ATPase in the endosome membrane (Mellman *et al.*, 1986). Upon exposure to a pH of 6.2 or below, the SFV spike protein undergoes several characteristic changes in conformation and interacts with the target membrane (Kielian, 1995; Kielian, 1993, and discussed in section 2.2.3 below). The acid conformation of the spike protein then triggers the fusion of the virus membrane with the endosome membrane, releasing the viral nucleocapsid into the cytoplasm to initiate infection. Important evidence to support this pathway has been provided by studies with various agents that act by raising the pH of the endosome above the critical threshold required to trigger fusion (Glomb-Reinmund and Kielian, 1998b; Kielian, 1995; Kielian, 1993; Strauss and Strauss, 1994). Fusion and infection by both SFV and SIN can be blocked specifically during the early stages of virus uptake by adding weak bases such as ammonium chloride and chloroquine, ionophores such as monensin, or by treating cells with specific inhibitors of the endosomal ATPase such as bafilomycin A (Glomb-Reinmund and Kielian, 1998b; Pérez and Carrasco, 1994). Inhibition by these agents could be “bypassed” by binding the virus to the plasma membrane and brief low pH treatment to trigger direct fusion with the cell plasma membrane

(Helenius *et al.*, 1980; White *et al.*, 1980). Virus strains with a lower pH dependence for membrane fusion were shown to be more sensitive to inhibition by these agents (Kielian *et al.*, 1984; Glomb-Reinmund and Kielian, 1998b; Glomb-Reinmund and Kielian, 1998a), in keeping with the prediction that a lower concentration would be required to raise endosomal pH above the critical threshold level. While further discussion of the entry pathway for SFV and SIN infection is outside the scope of this review, the reader is referred to (DeTulleo and Kirchhausen, 1998; Glomb-Reinmund and Kielian, 1998b; Kielian, 1995; Kielian, 1993; Strauss and Strauss, 1994; Brown and Edwards, 1992) for additional information and perspectives.

2.2.2. *In Vitro* Fusion with Liposomes

SFV fusion has been extensively studied using protein-free liposomes as target membranes, and using either content mixing assays (White *et al.*, 1980; Kielian *et al.*, 1996) or membrane mixing assays based on dilution of a fluorescent probe such as pyrene or octadecylrhodamine (Wahlberg *et al.*, 1992; Bron *et al.*, 1993). Some key points about SFV fusion with liposomes are that it is rapid (at 37 °C, fusion is complete within ~5sec), receptor-independent, very efficient (50–95% of input virus), and highly pH-dependent, with a threshold pH of ~6.2 for wt SFV. Fusion is induced by low pH treatment and can be arrested before completion by shift to neutral pH. The *in vitro* pH dependence of virus-liposome fusion was found to correlate with the *in vivo* pH dependence of virus fusion with the endosome membrane (Kielian *et al.*, 1984; Glomb-Reinmund and Kielian, 1998b; Glomb-Reinmund and Kielian, 1998a). Unlike viruses such as HIV-1 (Berger, 1997) and ALSV (Hernandez *et al.*, 1997), SFV fusion can clearly occur without requiring receptor interaction. Thus the primary role of the SFV-receptor interaction *in vivo* is to carry the virus into the endosome where it is exposed to low pH. SFV fusion is temperature dependent, but occurs across a wide temperature range, 2°–37°C, with a uniform activation energy suggestive of a consistent fusion mechanism at each temperature (Bron *et al.*, 1993). Strikingly, while receptors, calcium, ion and pH gradients do not seem to be required for SFV-liposome fusion, fusion is highly dependent on the presence of cholesterol and sphingolipid in the liposome membrane, as described in detail below.

2.2.3. Conformational Changes in the Virus Spike during Membrane Fusion

One experimental advantage of the low pH-triggered alphavirus fusion reaction is the simplicity of exposing virus to either a fusion-active low pH

Table 1
Summary of events during SFV low pH-induced fusion

Order of events	Assay
1. Acid treatment	
2. E2-E1 dimer dissociation	sucrose gradient sedimentation, co-immunoprecipitation
3. E2 alterations	trypsin sensitivity
E1 alterations	Ab epitope exposure (enhanced by cholesterol and sphingolipid)
E1 homotrimerization	trypsin resistance, gradient sedimentation, SDS- PAGE (enhanced by cholesterol and sphingolipid)
4. Lipid bilayer interaction	liposome coflotation (cholesterol-dependent)
5. Other unknown steps	observed as lag time after virus-lipid binding
6. Fusion	lipid mixing, content mixing (requires cholesterol and sphingolipid)

Events are numbered in the probable sequence of their occurrence during the fusion reaction. The multiple events listed under some numbers have not yet been separated kinetically. Modified from (Kielian, 1995).

environment or a fusion-inactive neutral pH environment. The fusion reaction can be slowed by using sub-optimal conditions of pH and temperature, thus enabling careful dissection of the spike protein conformational changes involved. While the precise order of the conformational changes is not completely clear, a great deal of useful information has been gained by the analysis of these types of virus fusion reactions. Our current understanding of the spike protein conformational changes induced by low pH exposure and their relationship to fusion is summarized as follows and in Table 1. The lipid dependence of these conformational changes will be discussed in section 5.1.

The first spike protein alteration observed after low pH exposure is a change in the normally very stable but non-covalent E2-E1 dimer interaction. The acid-treated dimer dissociates upon solubilization in non-ionic detergent, and a loss of E2-E1 co-immunoprecipitation and co-floitation on sucrose gradients is observed. This dimer dissociation appears critical for virus fusion, since virus mutants that have a more acidic pH threshold for dimer dissociation also have a more acidic pH threshold for the subsequent conformational changes in E1 and for membrane fusion (Glomb-Reinmund and Kielian, 1998a). The p62-E1 dimer requires a considerably lower pH to trigger dissociation compared to the mature E2-E1 dimer (Wahlberg *et al.*, 1989), and this increased dimer stability is responsible for the more acidic pH required to trigger the fusion of virus or spike protein mutants con-

taining uncleaved p62 (Salminen *et al.*, 1992; Lobigs and Garoff, 1990; Jain *et al.*, 1991).

The evidence suggests that once the E2-E1 dimer is dissociated by exposure to low pH, the E1 subunit then undergoes several distinct conformational changes that are independent of further interactions with E2. Previously masked E1 epitopes for monoclonal antibody (mAb) binding become accessible upon low pH treatment. We have recently mapped one such acid-specific epitope to residue 157 of E1, implying that this region of E1 becomes exposed during the conformational changes involved in fusion (Ahn *et al.*, 1999a). A second conformational change is the formation of a homotrimer of E1 subunits (Wahlberg and Garoff, 1992; Wahlberg *et al.*, 1992). While the SFV E1 homotrimer is not predicted to contain an α -helical coiled-coil, it is clearly very stable, being resistant to SDS treatment at 30°C and highly resistant to trypsin digestion. With similar kinetics as these E1 conformational changes, the virus binds to target membranes (Bron *et al.*, 1993) via the E1 subunit, presumably due to insertion of the fusion peptide (Klimjack *et al.*, 1994). Photolabeling studies have demonstrated that the E1 region containing the putative fusion peptide is inserted into the lipid bilayer under fusion-active conditions (D. Mayer, J. Corver, J. Wilschut, M. Kielian, and J. Brunner, unpublished results). There is a lag period following membrane interaction, which is assumed to reflect additional conformational changes and/or reorganization of the E1 subunits in the membrane. Membrane fusion then ensues, as detected by both lipid and content mixing assays.

A useful form of E1 for studies of conformational changes has been a truncated ectodomain fragment termed E1*. E1* is produced by proteinase K cleavage at a site close to the E1 transmembrane domain (Kielian and Helenius, 1985). It contains most of the extraviral domain of E1 but lacks the hydrophobic transmembrane domain, and is a water-soluble monomer not associated with E2. Similar to full-length E1, E1* efficiently undergoes acid-dependent conformational changes resulting in acid-specific mAb binding, E1* trimerization, and E1* membrane interaction (Klimjack *et al.*, 1994; Glomb-Reinmund and Kielian, 1998a). These studies indicate that the E1 ectodomain contains the E1 regions responsible for these conformational changes, and that further E2-E1 interactions are not involved in the fusogenic conformational changes in E1 once the dimer has dissociated.

Conflicting data exist on whether E1 undergoes its acid-dependent conformational changes before membrane insertion occurs. Analysis of the kinetics of these changes suggests that both epitope exposure and homotrimerization occur slightly before membrane binding (Bron *et al.*, 1993). In addition, both homotrimerization and epitope exposure can take

place in the absence of a target membrane (Wahlberg *et al.*, 1992; Kielian *et al.*, 1990). In contrast to these results, although both fusion and the EI conformational changes are inhibited by the presence of Zn^{2+} , dimer dissociation and membrane binding are unimpaired (Corver *et al.*, 1997). Studies of a mutation in the putative EI fusion peptide have shown that the mutation completely blocks both homotrimer formation and membrane fusion, strongly arguing that the EI homotrimer is required for fusion (Kielian *et al.*, 1996). Results with this mutant suggest that EI-membrane insertion and acid-epitope exposure are relatively independent of homotrimerization. Little information is available to indicate if the homotrimer formed in the absence of a target membrane, or the membrane binding seen in the absence of homotrimerization, represents fusion-active homotrimer or membrane binding, making it difficult to conclude whether blocks in one event can be used to establish the overall sequence of events. Thus, at this point the order of EI-membrane association versus EI conformational changes (or their co-incident occurrence) cannot be resolved.

2.3. Virus Exit Pathway and Requirements

The final steps in production of progeny virus involve the budding and release of completed virus particles at the plasma membrane of the host cell (Strauss and Strauss, 1994; Kielian, 1995; Garoff *et al.*, 1994; Strauss *et al.*, 1995). From mutagenesis studies of the SFV infectious clone, it is clear that SFV particle formation requires co-expression of both capsid and spike proteins (Suomalainen *et al.*, 1992). This is due to a specific interaction of the SFV E2 cytoplasmic tail with the nucleocapsid (Lee *et al.*, 1996; Skoging *et al.*, 1996), a reaction believed to drive budding. In contrast, the EI cytoplasmic tail is dispensable for virus production (Barth *et al.*, 1992). Amino acid residues across the entire E2 31 amino acid cytoplasmic tail are probably involved in nucleocapsid binding (Strauss and Strauss, 1994), and in particular a key tyrosine residue in a pentapeptide repeat region appears to be critical for both binding and particle production (Zhao *et al.*, 1994). The spike-nucleocapsid interaction appears to stabilize both the nucleocapsid structure (Forsell *et al.*, 1996) and the spike protein (Zhao and Garoff, 1992). A number of studies have also implicated the alphavirus 6K polypeptide in virus exit (Kielian, 1995). Deletion analysis of 6K in an SFV infectious clone demonstrated that it was not essential for normal spike protein dimerization and transport to the cell surface, or for normal virus structure and infectivity, but seemed to play a role in the efficient budding of virus from the cell (Liljeström *et al.*, 1991). Both the E2 and 6K proteins are modified by fatty acylation within their transmembrane and cytoplasmic domains, and mutations that disrupt acylation can also affect budding

(Gaedigk-Nitschko and Schlesinger, 1991; Gaedigk-Nitschko *et al.*, 1990; Ivanova and Schlesinger, 1993).

It is apparent that lateral spike-spike interactions are also critical for SFV assembly at the plasma membrane (Kielian, 1995). Cross-linking analysis shows that the spike protomer is organized as a trimer on the surface of infected cells (Rice and Strauss, 1982). Although the cellular site of spike trimerization is not clear, trimer interactions are important in virus exit. A non-budding spike protein mutant with an E2 tail negative for capsid binding could be rescued by the formation of mixed trimers with the wt spike protein (Ekstrom *et al.*, 1994). In addition, several examples indicate that E1-E2 dimer interactions are also critical for virus assembly and budding. The E2 cytoplasmic tail does not interact with the nucleocapsid unless the E2 subunit is complexed with E1 (Barth and Garoff, 1997). SFV spike proteins containing mutations within the putative E1 fusion peptide form highly unstable E1-E2 dimers (Duffus *et al.*, 1995). These mutant spike proteins are efficiently synthesized and transported to the plasma membrane where they associate with the nucleocapsid, but show a strong and thermoreversible defect in virus exit (Duffus *et al.*, 1995).

In summary, efficient alphavirus exit requires key viral components: the virus spike and nucleocapsid, the specific interaction of the E2 tail with nucleocapsid, the expression of 6K, and correct lateral spike protein interactions. The role of cholesterol in virus exit will be discussed in section 3.2 below.

3. THE ROLE OF CHOLESTEROL IN THE ALPHAVIRUS LIFECYCLE

3.1. Role of Cholesterol in Fusion

3.1.1. *In Vitro* Cholesterol Requirements

A role for cholesterol in alphavirus membrane interactions was first detected in studies of virus-liposome binding by Mooney *et al.* (Mooney *et al.*, 1975), who found that SIN bound to liposomes in a low pH and cholesterol-dependent reaction. As discussed above (section 2.2.3), such virus-liposome association is indicative of either E1-membrane interaction prior to fusion and/or the actual fusion reaction itself. Once the analysis of SFV endocytic uptake indicated that virus fusion was triggered by low pH, the *in vitro* fusion of SFV with liposomes was characterized using content mixing assays (White and Helenius, 1980). Fusion with liposomes composed

of a variety of purified lipids revealed a striking requirement for cholesterol in SFV membrane fusion. Fusion is highly efficient (~90%) with "complete" liposomes containing phosphatidylcholine(PC) : phosphatidylethanolamine (PE) : sphingomyelin(Sph) : cholesterol in a 1 : 1 : 1 : 1.5 ratio. In the absence of cholesterol, no fusion is observed, and maximal fusion requires a ratio of about 1 cholesterol per 2 phospholipid molecules. This cholesterol : phospholipid ratio is similar to those reported for the plasma membrane of animal cells (Dawidowicz, 1987), and thus these results suggested that the virus would show a similar cholesterol dependence during fusion with the host cell endosome membrane.

A later study used the SFV-liposome fusion system to examine the structural features of fusion-permissive sterols (Kielian and Helenius, 1984). Cholesterol causes a number of physical effects in membranes, including phospholipid condensation, increases in bilayer stability, decreased hydration, increases in the fluidity of the gel phase, and decreases in the fluidity of the liquid-crystalline phase (Nes and McKean, 1977a; Demel and DeKrijff, 1976; Kielian and Helenius, 1984). Three major features of cholesterol are believed to be important in its physical interactions with the membrane: the planar ring structure, the aliphatic side chain at C-17, and a free β -hydroxyl group at C-3 (Nes and McKean, 1977a; Demel and DeKrijff, 1976). SFV fusion occurred with coprostanol, a sterol containing a non-planar ring structure, and with androstanol, a sterol lacking the iso-octyl side chain. Dihydrocholesterol, which lacks the 5,6 double bond, was also fully active. In contrast, any sterol with a modified 3 β -hydroxyl group was inactive in fusion, including epicholesterol (3 α -hydroxy), cholestanone, 5 α -cholestane, cholesterol methyl ether, or cholesterol acetate (Kielian and Helenius, 1984), and chlorocholestene (Phalen, 1993). The sterol requirement for SFV fusion thus correlates with the sterol 3 β -hydroxyl group rather than with the known effects of sterol on the physical properties of the lipid bilayer. Virus-liposome binding experiments demonstrated that binding is both low pH and sterol-dependent (Kielian and Helenius, 1984; Wahlberg *et al.*, 1992). Interestingly, although epicholesterol was inactive in fusion, it could support virus-liposome binding at about 30% the level observed for cholesterol (Kielian and Helenius, 1984). The cholesterol dependence of both SFV-liposome fusion and binding is sigmoidal (White and Helenius, 1980; Nieva *et al.*, 1994), suggestive of some role of cooperativity such as formation of cholesterol-enriched domains (Nieva *et al.*, 1994).

Subsequent experiments using sensitive lipid mixing assays have corroborated the *in vitro* cholesterol requirement for SFV fusion and the importance of the 3 β -hydroxyl group (Bron *et al.*, 1993; Wahlberg *et al.*, 1992). Both lipid and content mixing assays thus demonstrate strong cho-

lesterol dependence, indicating that a cholesterol-deficient membrane is not competent to carry out "hemifusion", the mixing of the two membrane outer leaflets, a step that is believed to occur prior to complete fusion or content mixing. In addition, recent experiments on SIN fusion with liposomes indicate that it is also cholesterol dependent (personal communication from J. Smit and J. Wilschut).

3.1.2. *In Vivo* Cholesterol Requirements

Although the phospholipid and fatty acyl chain composition of the liposomes used in the above fusion assays was selected to be similar to that of a mammalian host cell, the possibility remained that the strong cholesterol dependence observed *in vitro* might not be relevant to the productive infection pathway. In order to address the role of cholesterol in both fusion with biological membranes and infection of a cell, we took advantage of the fact that insect cells are cholesterol auxotrophs (Nes and McKean, 1977b) and mosquito cells are productive hosts for SFV. As demonstrated by (Silberkang *et al.*, 1983), insect cells in culture can be grown in low density lipoprotein (LDL)-depleted serum for multiple passages to achieve virtually complete cholesterol removal in the absence of changes in phospholipid composition or synthesis of replacement sterol. Unlike the situation in mammalian cells, cholesterol-depletion of insect cells causes no deleterious effects. We cultured mosquito cells (the C6/36 line) in LDL-depleted serum to deplete them to non-detectable levels of cholesterol (Phalen and Kielian, 1991). We then used the C6/36 cells to compare the virus infection pathway under control and sterol-depleted conditions. Virus binding, endocytic uptake, and endosome acidification are unaltered in the cholesterol depleted cells, but virus fusion and infection are blocked. Quantitation of infection of cholesterol-depleted cells indicates that it is reduced by ~4 logs compared with control cells (Vashishtha *et al.*, 1998; Marquardt *et al.*, 1993). The efficiency of virus fusion with the plasma membrane of sterol-depleted cells is reduced by ~5 logs (Vashishtha *et al.*, 1998). In contrast, depleted cells are readily infected either by direct transfection of SFV RNA, or by vesicular stomatitis virus (VSV), an unrelated virus that also enters cells via endocytosis and low pH-mediated fusion but is not cholesterol-dependent (Marquardt *et al.*, 1993). Repletion of cells with cholesterol reversed the block in SFV infection, while cholestenone, which lacks the critical 3 β -hydroxyl group, was inactive (Phalen and Kielian, 1991). Recent work with SIN demonstrated that both SIN infection and fusion are highly cholesterol dependent, with a difference of about 5 logs between control and sterol-depleted cells (Lu *et al.*, 1999). Taken together, these results confirmed that, in keeping with the biochemical studies, cho-

lesterol plays an important role during *in vivo* fusion and infection by SFV and SIN.

3.2. Role of Cholesterol in Virus Exit

Results from the infectivity studies showed that cholesterol-depleted cells could be infected with SFV by infecting at high multiplicity or by direct transfection of the virus RNA. These methods were used to express the SFV genome in depleted cells and examine the cholesterol requirements for post-fusion steps in the virus life cycle (Marquardt *et al.*, 1993). The depleted cells transcribe virus RNA and translate the virus non-structural and structural proteins. The spike proteins are synthesized, p62 is cleaved, and the E1 and E2 spike subunits are transported to the plasma membrane. Thus most of the steps of the virus replication cycle appear largely unaffected by the absence of cholesterol. However, while the control cells efficiently release virus particles into the medium, the exit of SFV from depleted cells is dramatically inhibited, and is restored by repletion of the cells with cholesterol. The depleted cells are capable of budding virus particles, since VSV, which is cholesterol-independent for fusion, was efficiently produced in sterol-depleted cells (Marquardt *et al.*, 1993; Cleverley *et al.*, 1997). The cholesterol-dependence of SIN virus exit has recently been examined and was found to be dependent on sterol, analogous to the results with SFV (Lu *et al.*, 1999). Thus SFV and SIN are similar in their dependence on cholesterol for both entry (fusion) and exit. These two cholesterol requirements result in a strong inhibition of SFV and SIN growth in sterol-depleted cells compared to control cells, with final titers from 4–5 logs lower in the depleted cells (Vashishtha *et al.*, 1998; Lu *et al.*, 1999). As SIN and SFV are rather distantly related alphaviruses, their comparable cholesterol dependence suggests that sterol dependent fusion and exit are general properties of the alphavirus family. These data are in keeping with a previous paper suggesting that cells containing an increased level of cholesterol are resistant to inhibition of virus exit by low NaCl incubation (Garry *et al.*, 1985).

A partial exception to the cholesterol requirement *in vivo* has been described in mosquito cells depleted of cholesterol by prolonged passage in medium containing lipoprotein-deficient serum (Marquardt and Kielian, 1996). Following extended culture in the absence of lipoproteins, the cells “adapted” to sterol depleted conditions, and were then relatively more permissive for virus infection, fusion with the plasma membrane, exit, and growth. This adaptation is poorly characterized at present, but points out both the importance of limited passage of cells under sterol-depleting conditions, and the possibility that additional factors in the target membrane,

possibly lipids, may act to modulate the cholesterol dependence of SFV fusion and exit.

4. THE ROLE OF SPHINGOLIPID IN ALPHAVIRUS FUSION

4.1. *In Vitro* Requirement for Sphingolipid in Virus-Membrane Fusion

Studies from the laboratory of Jan Wilschut have demonstrated that the fusion of SFV with target liposomes is strikingly dependent on the presence of sphingolipid in the target membrane (Nieva *et al.*, 1994; Wilschut *et al.*, 1995; Corver *et al.*, 1995; Moesby *et al.*, 1995; He *et al.*, 1999). Both lipid and content mixing assays demonstrated strong sphingolipid dependence, indicating that sphingolipid is required for both hemifusion and complete fusion. About 2 mole percent sphingomyelin is sufficient to support maximal SFV liposome fusion (Nieva *et al.*, 1994), considerably lower than the ~30 mole percent cholesterol required for maximal SFV liposome fusion (White and Helenius, 1980). Sphingolipid does not seem to be required for the initial hydrophobic interaction of the virus with the target membrane (Nieva *et al.*, 1994; Moesby *et al.*, 1995), while efficient virus-liposome interaction does require cholesterol (Kielian and Helenius, 1984; Wahlberg *et al.*, 1992) (Table I). Recent experiments on SIN fusion with liposomes indicate that this virus is also strongly sphingolipid dependent (personal communication from J. Smit and J. Wilschut). Fusion studies with a wide variety of sphingolipids have demonstrated an interesting requirement for distinct structural features of the sphingolipid molecule, as detailed below. Thus both the cholesterol and sphingolipid requirements are suggestive of specific lipid-protein interactions during fusion.

4.2. Structural Features of Fusion-Permissive Sphingolipids

Sphingomyelin, ceramide, and galactosyl ceramide are all active in SFV fusion, indicating that the headgroup of the sphingolipid is not critical for fusion and can be either phosphocholine, a hydroxyl group, or a carbohydrate moiety (Nieva *et al.*, 1994). Ceramide is the minimal sphingolipid that can support fusion, and the sphingosine base is inactive (Nieva *et al.*, 1994). The reaction is stereospecific for the naturally occurring D-erythro stereoisomer while the three unnatural stereoisomers of ceramide are inactive (Moesby *et al.*, 1995). Acyl chain length does not appear critical since both C18-ceramide and C8-ceramide are active (Corver *et al.*, 1995; Moesby *et al.*, 1995). 3-deoxyceramide or 3-methoxyceramide do not support fusion, demonstrating the importance of the 3-hydroxyl group on the sphingosine

backbone (Wilschut *et al.*, 1995; Corver *et al.*, 1995). The 4,5-trans carbon-carbon double bond of the sphingosine backbone is also important, since dihydroceramide and $\Delta 5$ -trans ceramide are inactive (Corver *et al.*, 1995; He *et al.*, 1999). Although cholesterol and sphingomyelin are known to form complexes, complex formation does not appear to be required for fusion, since galactosyl ceramide is fusion-active but does not efficiently interact with cholesterol, at least in monolayer experiments (Nieva *et al.*, 1994).

Although sphingolipid synthesis inhibitors have been described (Rosenwald *et al.*, 1992), their prolonged use is toxic to tissue culture cells, and short-term use does not deplete cells below the level of sphingolipid required for fusion. As there are to date no viable sphingolipid-deficient cell lines, it has not yet been possible to examine the role of sphingolipids *in vivo* during alphavirus fusion and exit.

5. MECHANISMS OF CHOLESTEROL AND SPHINGOLIPID REQUIREMENTS IN ALPHAVIRUS FUSION AND EXIT

5.1. The Role of Cholesterol and Sphingolipid in Fusogenic Spike Protein Conformational Changes

The first evidence for a role of cholesterol in the SFV fusogenic conformational changes came from studies of the El* ectodomain (Kielian and Helenius, 1985). El* only converts to its trypsin-resistant form when treated at low pH in the presence of liposomes containing cholesterol (PC:PE:Sph : cholesterol). Epicholesterol-containing liposomes at either acid or neutral pH or cholesterol liposomes at neutral pH are inactive in supporting this conformational change. Subsequent experiments demonstrated that El* is similarly dependent on cholesterol for conversion to reactivity with acid-conformation-specific mAbs (Kielian *et al.*, 1990), and for El*-membrane binding and El* trimerization assayed by SDS-PAGE (Klimjack *et al.*, 1994). Thus these experiments are all consistent in their requirement for a fusion-active sterol in the conformational changes believed to reflect the fusion-active form of El. Studies of isolated lipid-free spike protein rosettes also show that acid epitope exposure and El trimerization are more efficient in the presence of cholesterol liposomes, although some conversion also occurs using liposomes without sterol (Justman *et al.*, 1993).

In contrast, studies of the low pH-dependent conformational changes in full length viral El did not reveal a requirement for cholesterol in these conformational changes (Kielian and Helenius, 1985; Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Justman *et al.*, 1993; Kielian, 1995). Recent experiments have demonstrated that this apparent difference between El* and viral El

reflects the fact that although the conformational changes in full length EI can occur in the absence of sterol, their kinetics are significantly enhanced when the virus is acid-treated in the presence of cholesterol liposomes (Corver, 1998). The early kinetics of the viral EI conformational changes were examined using sub-optimal pH and/or temperature to slow the fusion reaction and allow careful kinetic measurements. These studies show that EI homotrimerization (Corver, 1998; Chatterjee and Kielian, 1999b), and mAb epitope exposure (Chatterjee and Kielian, 1999b) are all strikingly potentiated by the addition of cholesterol-containing liposomes, and not by sterol-depleted liposomes. In contrast, dimer dissociation appears not to require the presence of a target membrane (Wahlberg *et al.*, 1989), in keeping with its being one of the first steps in the fusion pathway, prior to the subsequent EI conformational changes (Justman *et al.*, 1993; Corver *et al.*, 1997).

Similar to cholesterol, the initial suggestion that sphingolipids play a role in EI conformational changes came from studies with EI* (Klimjack *et al.*, 1994). Although not as absolute as the cholesterol dependence of EI*, ectodomain homotrimerization, acid epitope exposure, and liposome binding are all greatly enhanced by the presence of sphingolipid in the target membrane. However, even examination of the early kinetics of the conformational changes in full length viral EI using liposomes containing cholesterol, PC, PE with or without Sph found no effect of sphingolipid (Corver, 1998). Homotrimer formation was then evaluated using cholesterol-minus liposomes with or without sphingolipid (PC: PE : Sph vs. PC: PE) (Corver, 1998). This experiment was feasible since, as described above, cholesterol-depleted liposomes support EI conformational changes although at a slower rate. Under these cholesterol-depleted conditions, a strong enhancement of EI homotrimerization by sphingolipid is observed (Corver, 1998). This enhancement was confirmed for both EI homotrimerization and acid epitope exposure (Chatterjee and Kielian, 1999b). Interestingly, the enhancement of EI conformational changes by sphingolipid thus takes place using a sterol-depleted target membrane that is negative for both virus binding and fusion.

The above results indicate the importance of assaying the kinetics of spike protein conformational changes within the time frame of the fusion reaction, when rate-limiting steps in fusion can be differentiated (Bron *et al.*, 1993; Justman *et al.*, 1993). Taken together, these results suggest that EI must interact with the target membrane at a step in fusion prior to EI homotrimerization and epitope exposure, so that the presence or absence of the critical lipids is detected by EI and reflected in the kinetics with which these conformational changes occur. Such a "lipid-sensing" interaction would presumably occur earlier and differ from the stable insertion

of the EI fusion peptide into the membrane detected by liposome co-floitation. The existence of a discrete “lipid-sensing” step is supported by the fact that enhancement of EI conformational changes by sphingolipid is observed even using cholesterol-deficient liposomes that are negative for EI-fusion peptide insertion. A key question is to identify the domain(s) of EI that are responsible for its interactions with cholesterol and sphingolipid during fusion. The suggestion from these results is that these domain(s) may be distinct from the viral fusion peptide.

5.2. Alphavirus Mutants with Reduced Cholesterol Requirements

To gain a better understanding of the role of cholesterol in SFV fusion and exit, and to identify specific spike protein domains involved in the cholesterol interaction, our laboratory has used several strategies to isolate SFV mutants that are significantly independent of cholesterol. One of these mutants, *srf-3*, has been extensively characterized and the results are summarized here. Further work focuses on the characterization of the region defined by this mutation and the identification of other regions and residues of the EI spike protein that are important for determining the cholesterol requirement in the alphavirus lifecycle.

5.2.1. Sequences Involved in the Alphavirus Cholesterol Requirement

5.2.1.1. Sequences Involved in the SFV Cholesterol Requirement.

SFV cholesterol-independent mutants were derived by selecting for growth on cholesterol-depleted cells, thus simultaneously applying a selection pressure for decreased cholesterol dependence of both the fusion and exit steps of the virus lifecycle (Vashishtha *et al.*, 1998; Marquardt *et al.*, 1993; Chatterjee and Kielian, 1999a). Such selections have been performed using mutagenized or non-mutagenized virus stocks, and growth on either cholesterol-depleted mosquito cells or depleted cells repleted with a non-fusogenic cholesterol analogue, chlorocholestene. All of the resultant mutant viruses have a similarly increased ability to grow on cells lacking cholesterol. In all cases to date, however, the mutants still grow more efficiently on cells containing cholesterol than on cells without sterol. The first three mutant isolates were termed *srf-1*, -2, and -3, for sterol requirement in function (Vashishtha *et al.*, 1998; Marquardt *et al.*, 1993). Sequence analysis (discussed below) revealed that all three have the same genotype (Vashishtha *et al.*, 1998). Much of the subsequent characterization has been performed with *srf-3*.

Phenotypic analysis of the *srf-3* virus reveals that it has growth kinetics comparable to wt SFV when assayed on either BHK cells or on mos-

quito cells containing cholesterol (Vashishtha *et al.*, 1998). In contrast, although *srf-3* grows more slowly on cholesterol-depleted cells than control cells, it achieves the same final titer on both cell types, with an overall yield four to five logs higher than that produced by wt SFV in depleted cells (Vashishtha *et al.*, 1998). This enhanced growth on sterol-depleted cells is due to an increase in both the entry and exit of *srf-3*. Infection and fusion with cholesterol-depleted cells are increased at least 2 logs in *srf-3* compared with wt SFV. Pulse-chase experiments demonstrate that *srf-3* shows more efficient exit from sterol-depleted cells compared to wt SFV (Marquardt *et al.*, 1993; Vashishtha *et al.*, 1998; Marquardt and Kielian, 1996). Transmission electron microscopy indicates that *srf-3* virus buds from the plasma membrane of cholesterol-depleted cells and has an overall morphology similar to wt virus or *srf-3* produced in control cells (Marquardt *et al.*, 1993; Vashishtha *et al.*, 1998). The cholesterol-independent phenotype of *srf-3* is stable to passage in cholesterol-containing mosquito cells or BHK cells (Vashishtha *et al.*, 1998), or to passage in the mosquito vector (Ahn *et al.*, 1999b). Quantitation of primary infection demonstrates that the *srf-3* mutation does not change the host range of the virus on cholesterol-containing mosquito cells or BHK cells (Vashishtha *et al.*, 1998). Thus, the *srf-3* phenotype is similar to that of wt virus except for its increased infection, fusion, and exit in cholesterol-depleted cells.

Further *in vivo* analysis was performed with *srf-3* to see if its relative cholesterol-independence would affect its growth in mosquitoes, which like all insects are cholesterol auxotrophs (Ahn *et al.*, 1999b). *srf-3* was found to grow more efficiently than wt virus in mosquitoes. The difference is not as marked as the growth advantage of *srf-3* in sterol-depleted cells, and it is not clear if the increased *srf-3* virus production in the mosquito is related to the availability of cholesterol in the host.

Interestingly, an additional effect of cholesterol on virus particle stability has also been observed (Vashishtha *et al.*, 1998). *srf-3* virus propagated in cholesterol-depleted cells is less stable to centrifugal shear force than wt or *srf-3* propagated on cholesterol-containing cells. This result suggests a possible role of cholesterol in stabilizing spike protein interactions involved in the alphavirus particle.

Given what was known about SFV fusion and the effect of cholesterol on low pH-dependent conformational changes, the prediction was that the *srf-3* mutation would map to the E1 subunit. Mapping studies and sequence analysis revealed a single point mutation in the *srf-3* E1 spike protein, a change of proline at position 226 to serine (P226S) (Vashishtha *et al.*, 1998) (Figure 3). Introduction of this mutation *de novo* into the SFV infectious clone conferred the cholesterol-independent phenotype in SFV entry/fusion and exit, confirming the identity of the critical mutation. Our current understanding of the effect of this mutation on the conformational changes

<u>Virus</u>	224				<u>Sterol independence</u>
SFV	PSPGM	VHVPY	TQ		-
<i>srf-3</i>	-- S --	-----	--		+
SIN	--AKN	-----	--		-
<u>SKN</u>	-- SKN	-----	--		-
<u>SGM</u>	-- SGM	-----	--		+
<u>SGN</u>	-- SGN	-----	--		+
<u>SKM</u>	-- SKM	-----	--		-
<u>AGM</u>	-- AGM	-----	--		+
<u>PGM</u>	-- PGM	-----	--		-

FIGURE 3. The sequence of SFV, *srf-3*, SIN, and several SIN mutants in the EI region from amino acids 224–235. The full sequence of SFV is listed, and the single amino acid change at residue 226 in *srf-3* is listed in bold while identical amino acids are indicated as dashes. The SIN sequence differences from SFV are listed while sequence identities are indicated as dashes. The changes from the SIN sequence in the SIN mutants are shown in bold in the sequence and underlined in the mutant name. Increased virus sterol independence for fusion is indicated as a +.

in *srf-3* is discussed below. It will now be important to determine if other regions of the SFV spike protein and other residues in the P226 region can similarly affect virus cholesterol dependence. Recently, two new *srf* mutants, *srf-4* and *srf-5*, have been isolated and shown to have increased infectivity, fusion, and growth on sterol-depleted cells, similar to *srf-3* (Chatterjee and Kielian, 1999a). The *srf-4* and -5 mutations are located at position 44 and 178 on the EI subunit, respectively. Thus it is already clear that additional regions of EI can affect cholesterol dependence. These sequences and their potential interactions are currently being explored.

The EI P226S mutation has been independently isolated 8 times using several different selection strategies, emphasizing the overall importance of this residue and region in SFV cholesterol dependence (Vashishtha *et al.*, 1998; Chatterjee and Kielian, 1999a). We have used *in vitro* mutagenesis of the SFV and SIN infectious clones to explore the role of the P226 region in virus cholesterol dependence. Results for SIN are discussed in (Lu *et al.*, 1999) and in section 5.2.1.2 below. Mutagenesis of the SFV infectious clone is being used to introduce various single point mutations into the EI 226 region and test the progeny virus phenotype (Gibbons and Kielian, 1999). Because no other alphaviruses have a proline at position 226, our initial hypothesis was that the gain of the serine, rather than the loss of the proline, accounted for the mutant phenotype (Vashishtha *et al.*, 1998). Reasoning that the hydroxyl group on the side chain of serine might be acting to

somehow substitute for the 3 β -OH of the cholesterol, the residue was mutated to a threonine. However, in changing the residue to a threonine (P226T) the wildtype phenotype was seen, whereas changing it to an alanine (P226A) produced a *srf-3* phenotype for both fusion and infection (Gibbons and Kielian, 1999). These results suggest that our initial hypothesis was probably an oversimplification, and that a hydroxyl-containing side chain is not specifically required at El position 226. Instead, our data in both the SFV and SIN systems (Lu *et al.*, 1999) indicate that the overall conformation of the El 226 region is important in modulating the virus cholesterol dependence.

5.2.1.2. Sequences Involved in the SIN Cholesterol Requirement.

Our data indicate that the region around El residue 226 is involved in the cholesterol dependence of SFV entry and exit. This region of El is quite conserved among alphaviruses over a length of 12 residues (Figure 3), but position 226 itself is not conserved, and can be either proline (SFV and Ross River virus), valine (western equine encephalitis virus), or alanine (remaining alphaviruses) (Vashishtha *et al.*, 1998). In SIN, the sequence of El from residue 224–235 is identical to that of SFV except for ²²⁶alanine-lysine-asparagine (AKN). In order to test the role of this region in SIN sterol-dependence, various SIN mutants were created (Figure 3), including a single point mutation changing SIN alanine to serine (SKN), and a triple mutation changing the wt SIN AKN sequence to SGM (SGM), thus making the sequence identical to that of *srf-3* from El 224–235 (Lu *et al.*, 1999). The SKN mutant is still strongly dependent on cellular cholesterol for its fusion, infection and growth. In contrast, the SGM mutant shows significantly enhanced infection and fusion with cholesterol-depleted cells compared to wt SIN, with increases of ~ 100 fold and 250 fold, respectively. Pulse-chase analysis of virus exit shows comparable kinetics for wt SIN and SGM in cholesterol-containing cells. In sterol-depleted cells, almost no wt virus is released, while the SGM mutant shows efficient virus exit. Thus, analogous to its role in SFV, the El 226 region acts in the control of the cholesterol dependence of both SIN entry and exit.

The importance of a specific amino acid or combination of amino acids in the control of SIN cholesterol dependence was investigated using several additional mutations in the El 226 region (Lu *et al.*, 1999) (Figure 3). Both the SGN and AGM mutants show increased cholesterol independence, indicating that serine²²⁶ and methionine²²⁸ are not specifically required. The SKM mutant is cholesterol-dependent for infection, indicating that a lysine at position 227 can abrogate the cholesterol independence of the SGM mutant. The PGM mutant is also strongly dependent on cholesterol, as might be predicted from the fact that it has the wt SFV sequence from position 224 to 235 on the SIN background. Thus, the overall conclusion from

these experiments is that the *srf-3* mutation of SFV predicts a region of EI involved in the cholesterol dependence of SIN. This region appears to act by affecting the conformation of EI, rather than by a simple role of specific amino acid side chains.

5.2.2. Mechanism of the *srf-3* Mutation

We hypothesized that the increased ability of the *srf-3* spike protein to fuse with cholesterol-depleted cell membranes is due to an alteration in some aspect of the EI spike protein's interaction with cholesterol, resulting in the relative cholesterol-independence of a normally cholesterol-requiring step in fusion. To test this hypothesis, the initial kinetics of the EI conformational changes were followed in the presence of either control or cholesterol-depleted liposomes using either wt or *srf-3* (Chatterjee and Kielian, 1999b). *srf-3* is strikingly less cholesterol-dependent than wt SFV for both acid-specific epitope exposure and EI homotrimer formation. At time points when wt EI conversion is greatly reduced in the absence of cholesterol, *srf-3* showed comparable levels of EI conversion in the presence or absence of cholesterol. These EI conformational changes were unaffected by the presence or absence of cholesterol in the virus membrane, as demonstrated by using *srf-3* stocks grown in either BHK cells or cholesterol-depleted mosquito cells.

In contrast, the *srf-3* EI conformational changes are still dependent on the presence of sphingolipid in the target bilayer (Chatterjee and Kielian, 1999b). Acid-specific epitope exposure and homotrimer formation were evaluated using sterol-free liposomes, and neither wild type nor mutant virus support efficient EI conversion in the absence of sphingolipid. Studies of virus infection in the presence of NH_4Cl demonstrate very similar NH_4Cl sensitivities for wt and *srf-3*, indicating that the two viruses have a comparable pH-dependence for fusion (Chatterjee and Kielian, 1999b). Thus, the *srf-3* mutant appears unaltered in its sphingolipid dependence and pH dependence, but is less dependent on cholesterol for the fusogenic conformational changes in EI.

The properties of wt and *srf-3* fusion were assayed *in vitro* in lipid mixing assays with pyrene-labeled wt or *srf-3* virus (Chatterjee and Kielian, 1999b). The initial rates of fusion were followed at different temperatures using complete liposomes (PC:PE:SPM:Chol. 1:1:1:1.5), and showed similar kinetics and extent of fusion for the two viruses. An Arrhenius diagram of initial fusion rates versus reciprocal temperature (Bron *et al.*, 1993) gave comparable plots for the two viruses, suggesting equivalent activation energies and general fusion characteristics between the two viruses. In contrast, when fusion with cholesterol-free liposomes (PC:PE:SPM 1:

1 : 1) was analyzed, significantly higher levels of fusion were observed with *srf-3* compared to wt virus. Fusion of both wt and *srf-3* showed comparable sphingolipid dependence. Thus, studies of virus interactions with defined lipid bilayers indicated that both the membrane fusion activity of *srf-3* and the low pH-dependent conformational changes in its E1 protein are relatively independent of cholesterol while being otherwise similar to those of wt SFV.

5.3. Mechanism of Cholesterol in Virus Exit

From the data summarized above, it is clear that both SFV and SIN need cholesterol for virus fusion and exit, and that mutations within the E1 226 region can modulate the cholesterol requirements for both viruses. Both the SFV mutant *srf-3* and the SIN mutant SGM which are less cholesterol dependent for virus entry are also less cholesterol dependent for virus exit, suggesting that the cholesterol requirement for alphavirus fusion may be tightly associated with its requirement for exit. Recent studies of SFV and *srf-3* indicate that the cholesterol dependent exit step(s) is late in the exit pathway, following arrival of the spike protein at the plasma membrane (Lu and Kielian, 1999). The exit pathway does not appear to require the virus fusion reaction per se, since the final virus assembly and budding steps involve membrane fission, a pinching-off reaction that initiates at the cytoplasmic surface of the plasma membrane and occurs at neutral pH (Marquardt *et al.*, 1993; Vashishtha *et al.*, 1998). Thus, while the data to date suggest a connection between the cholesterol dependence of alphavirus fusion and exit, the relationship between the cholesterol-dependent steps in fusion and exit, and the tightness of the linkage between them, is as yet unclear. One model is that the cholesterol requirements reflect a role of cholesterol in the optimal spike protein conformation for both fusion and exit. In the absence of cholesterol, mutations in the spike protein (e.g., within the E1 226 region) may act to compensate for the suboptimal spike conformation and thus allow efficient fusion and exit. The same regions of the spike protein that require cholesterol to potentiate fusogenic conformational changes may also be involved in interactions between spike proteins during virus assembly and exit. Future studies must address the role of cholesterol in the spike protein conformation and interactions, and their importance to the exit pathway.

6. ROLE OF SPECIFIC LIPIDS IN THE ENTRY AND EXIT OF OTHER PATHOGENS

Specific lipid requirements for membrane fusion and exit were first described for alphaviruses. Evidence is accumulating that particular lipids

may be involved in the entry or exit of other pathogens from cells, or in other fusion reactions. We will discuss a number of possible examples of such specific lipid requirements. In cases such as the cholesterol-binding toxins involved in bacterial entry, the data are compelling and informative at the molecular level. In other cases, the information available to date is incomplete and less reliable. The examples presented are not comprehensive and certainly include a wide range of approaches, methods, and some conflicting results. Nevertheless, they serve to illustrate some of the diverse ways in which other viruses or pathogens may use specific lipids as critical features of their lifecycles.

6.1. The Role of Cholesterol in Bacterial Toxin-Membrane Interactions

Clear examples of organisms that exploit cholesterol-protein interactions in their lifecycles are bacteria that produce what are termed thiol-activated cytolysins. The thiol-activated cytolysins are an antigenically and structurally related family of more than 20 toxins (including perfringolysin O, listeriolysin O, pneumolysin, and streptolysin O) that are produced by Gram-positive bacteria and are important virulence factors in bacterial pathogenesis (reviewed in Alouf and Geoffroy, 1991; Cossart and Mengaud, 1989). The toxins are protein monomers of 50–80 kDa that bind to cholesterol-containing membranes and oligomerize to form pores responsible for the permeabilization of cell membranes. Pores are estimated to contain ~50 monomers and to have a diameter of approximately 150Å. It was shown many years ago that toxin binding to cell membranes (or artificial membranes) requires cholesterol or a structurally related sterol with a 3 β -hydroxyl, that binding can be irreversibly inhibited by nM amounts of cholesterol in solution or by cholesterol-binding polyene antibiotics, and that prokaryotic or mycoplasma cells without cholesterol in their membranes are not susceptible to the lytic effects of the toxins (Alouf and Geoffroy, 1991; Watson and Kerr, 1974; Rottem *et al.*, 1976). The biological function of a thiol-activated cytolysin is probably best understood for the example of the facultative intracellular bacterium *Listeria monocytogenes* (reviewed in Portnoy *et al.*, 1992; Falkow *et al.*, 1992). *Listeria* are internalized by host cell phagocytosis, escape from the phagocytic vacuole, and replicate in the cytoplasm. Escape from the phagosome is mediated by bacterial secretion of the toxin listeriolysin O, which permeabilizes the vacuole by binding phagosome membrane cholesterol upon exposure to the acid pH of the phagocytic vacuole (Portnoy *et al.*, 1992). Listeriolysin-cholesterol binding has a pH optimum of 5.5, and requires the presence of a sterol with a 3 β -hydroxyl group. Thus, both alphaviruses and *Listeria* use low pH-dependent lipid interaction as a strategy to breach the plasma membrane barrier. While other thiol-activated cytolysins are also specific for 3 β -hydroxy sterols,

in general they do not have an acid pH optimum, suggesting that they act at the plasma membrane rather than within the endocytic system. This is in keeping with their role as lytic agents specific for cholesterol-containing cells.

The domain of the thiol-dependent cytolysins responsible for cholesterol binding is suggested to be localized to the most highly conserved sequence of the various proteins, the region containing a conserved cysteine from which this protein family derives its misplaced name. Although modification of the cysteine residue by various thiol-reacting reagents has long been known to result in loss of activity, this is probably due to steric hindrance of cholesterol binding, since site-directed mutagenesis shows that the cysteine is not necessary for toxin activity (904). A high resolution crystal structure of a perfringolysin O monomer (without bound cholesterol) was recently reported and was further used to build an homology model of pneumolysin (Rossjohn *et al.*, 1997; Rossjohn *et al.*, 1998). Sterol binding is believed to occur within domain 4 of the molecule, and to involve hydrogen bonds with the sterol 3 β -hydroxyl group and non-contiguous aliphatic side chains that interact with the sterol ring structure (Rossjohn *et al.*, 1997). Domain 3 is suggested to transverse the membrane during pore formation by virtue of a two-stranded antiparallel β -sheet (Shepard *et al.*, 1998). It appears that insertion of domain 3 into the membrane can occur with oligomerization-negative mutants (Shepard *et al.*, 1998). Studies of pneumolysin in the absence of membranes show that the toxin has the ability to dimerize and oligomerize in solution without cholesterol (Gilbert *et al.*, 1998). This recent data suggests that cholesterol is not absolutely required for the oligomerization step, although it increases the efficiency of the reaction significantly. In support of this latter point, fluorescence measurements of the conformational changes in streptolysin O suggest that binding to cholesterol-containing membranes facilitates an allosteric transition which initiates the oligomerization reaction (Palmer *et al.*, 1998). Despite all of this recent molecular work it is still unclear exactly how toxin monomers interact with cholesterol, whether the membrane-bound oligomer requires cholesterol, and what the precise structural changes are that result in either oligomerization and/or membrane insertion. These data are also interesting in light of the data indicating that SFV fusion peptide insertion requires cholesterol (Klimjack *et al.*, 1994; D. Mayer, J. Corver, J. Wilschut, M. Kielian, and J. Brunner, unpublished results) and is independent of oligomerization (Kielian *et al.*, 1996; Corver *et al.*, 1997), and the finding that effects on the virus cholesterol requirement are mediated by mutations in E1 outside of the fusion peptide (Vashishtha *et al.*, 1998; Chatterjee and Kielian, 1999a). The potential parallels between the role of cholesterol in the actions of bacterial toxins and in the membrane binding

and homotrimerization reaction of SFV are striking. Studies of these two systems may provide useful insights into common general features of protein-cholesterol interaction, oligomerization, and membrane insertion. It is also interesting that a hemolytic toxin from earthworm was recently reported to specifically bind sphingomyelin (Yamaji *et al.*, 1998), suggesting that other lipid-binding proteins remain to be characterized.

6.2. Other Viruses that May Require Specific Lipids

The majority of enveloped viruses have not yet been tested for lipid requirements in fusion, infection, or exit. This reflects the fact that for many viruses, both fusion and budding are poorly understood. In addition, the trigger for fusion of many viruses is more complicated than the low pH involved in triggering alphavirus fusion, making it difficult at present to study fusion in an *in vitro* system resembling the fusion reaction *in vivo*. The low pH-dependent fusion reactions of both influenza virus, an orthomyxovirus, and vesicular stomatitis virus (VSV), a rhabdovirus, have been well characterized *in vitro*, and both appear to have fusion mechanisms independent of cholesterol, sphingolipid, or other specific lipid components (White *et al.*, 1982; Eidelman *et al.*, 1984; Phalen and Kielian, 1991; Cleverley *et al.*, 1997; Stegmann and Helenius, 1993). Here we will discuss several examples of viruses that may have specific lipid interactions during either fusion or exit.

6.2.1. Human Immunodeficiency Virus

Studies of the retrovirus human immunodeficiency virus (HIV-1 and HIV-2) have shown that the lipid composition of its envelope appears significantly different from that of the host cell plasma membrane, with the virus membrane containing a lower total lipid/protein ratio, a higher cholesterol/phospholipid ratio, and substantial changes in the phospholipid class ratios (Aloia *et al.*, 1993; Aloia *et al.*, 1988). Electron spin resonance studies suggest that the virus membrane lipid composition produces a more ordered membrane in the virus compared to the host cell plasma membrane from which it buds (Aloia *et al.*, 1988). Treatment of virus with cholesterol-poor liposomes, cholesterol-binding drugs, or heating all increase the membrane fluidity and decrease virus infectivity (Pleskoff *et al.*, 1995; Aloia *et al.*, 1988; McDougal *et al.*, 1985). These data suggest that HIV may selectively exit from regions of the host cell plasma membrane enriched in cholesterol and sphingomyelin, and that the fluidity of the viral membrane may be important in virus stability and infectivity. However, the data are complicated by our current technical limitations in preparing purified plasma

membranes, which make it difficult to conclude what differences in lipid composition exist between HIV and host cell plasma membranes. The precise structural or functional requirements for cholesterol during HIV exit from the target cell, and the ability of other sterols to substitute thus are as yet unclear.

The role of target membrane cholesterol in HIV fusion has been tested by assaying the *in vitro* fusion of liposomes by peptides with sequences derived from the N-terminal fusion peptide of the HIV gp41 fusion protein. These studies demonstrated that the absence of cholesterol in the liposomes inhibits peptide-induced fusion by ~33%, while peptide-membrane binding is unaffected (Pereira *et al.*, 1997a; Pereira *et al.*, 1997b). Peptide-dependent fusion is inhibited by several physiologically relevant modulators of HIV-1 infectivity, such as inclusion of point mutations in the peptide that block HIV fusion, competition by hexapeptides derived from the N-terminal gp41 fusion peptide, and addition of several antiviral compounds. These results suggest that the peptide fusion system may accurately reflect an *in vivo* role for cholesterol in the target membrane during HIV fusion. However, it is important to note that such peptide-liposome fusion studies are prone to possible artifacts and may not reflect the mechanism of biological membrane fusion (Stegmann *et al.*, 1989a). For example, studies with influenza virus have demonstrated that the membrane insertion observed with a purified virus peptide from the spike coiled-coil region (Yu *et al.*, 1994) does not occur during the actual fusion of influenza virus with a target membrane (Durrer *et al.*, 1996). In addition to the potential problems with peptide-liposome studies, a group using an *in vitro* virus-liposome fusion system found that the presence or absence of cholesterol does not affect the kinetics or efficiency of fusion of HIV-1 or the closely related SIV-1 (Larsen *et al.*, 1990; Larsen *et al.*, 1993). The exact physiological relevance of the latter study is also not clear, since virus fusion in this system occurred with target membranes not containing the CD-4 receptor and co-receptor, the key molecules known to trigger HIV fusion during infection (Berger, 1997; Binley and Moore, 1997). Thus, a better understanding of the role of cholesterol in HIV entry and exit awaits further study.

The effects of cholesterol on HIV fusion are believed to be exerted via the virus fusion protein subunit gp41, which contains the putative fusion peptide at its N-terminus. The HIV gp120 spike protein subunit is responsible for the binding of virus to cells, usually by recognition of the CD4 receptor. However, several groups have identified the glycosphingolipid galactosylceramide (GalCer) as an alternative receptor to account for infection of CD4-negative cells such as colonic epithelial cells, Schwann cells, and oligodendrocytes (Harouse *et al.*, 1991; Yahi *et al.*, 1992). Infection of cultured human colonic epithelial or neural cells is blocked by antibodies

against GalCer. In addition, the binding of recombinant gp120 to purified GalCer can be specifically inhibited by an anti-GalCer IgG. Binding of purified gp120 to GalCer in a liposome system is rapid, saturable, extremely stable, dependent on a critical GalCer concentration in the target membrane, and blocked by antibodies to GalCer (Long *et al.*, 1994). This interaction is also dependent on the conformation of the gp120. Various other structurally related glycosphingolipids (GSLs) such as lactosylceramide, glucosylceramide, and galactocerebroside sulfate can also bind purified gp120, but with decreased efficiency and stability. Several recent reports have also demonstrated that the addition of a GSL fraction to CD4 positive cells resistant to HIV-1 infection confers susceptibility to viral fusion, suggesting a role for GSLs as possible co-receptors for binding virus (Puri *et al.*, 1998a; Puri *et al.*, 1998b). The active component of the GSL fraction in conferring fusion activity in resistant cells is the neutral GSL, Gb3 (Puri *et al.*, 1998a). The relative *in vivo* importance of such GSL as alternative HIV receptors or coreceptors is currently under investigation. Taken together, the above results suggest that for HIV, as with alphaviruses, different target membrane lipid components may play a role at different stages of the virus lifecycle.

6.2.2. Mouse Hepatitis Virus

Mouse hepatitis virus (MHV) is a murine coronavirus that usually induces a persistent infection of the central nervous system, but many strains may also cause an acute, lytic infection marked by significant cell-cell fusion (Wege *et al.*, 1983). The choice between persistent and lytic infection is partially determined by the host cell and appears to be influenced by the cholesterol content of the cell membrane. Studies with cultured mouse fibroblast sublines, either susceptible or relatively resistant to virus-mediated cell fusion, showed that supplementation with cholesterol increases the membrane cholesterol/fatty acid ratio and concomitantly increases cell fusion in both cell types (Daya *et al.*, 1988; Cervin and Anderson, 1991). This effect is not due to enhanced MHV internalization, but rather due to some late event in infection, resulting in enhanced syncytia formation. In fact, it was shown that increased cholesterol in target membranes enhanced fusion between infected and neighboring uninfected cells. Further work demonstrated that changes in cholesterol content alone account for the enhancement of fusion because no alterations in the levels of fatty acids were detected. As previously discussed (Kielian, 1993; Kielian, 1995), virus-induced cell-cell fusion, although widely used and frequently useful, is a morphological assay that can be affected by numerous factors not directly relevant to the virus infection pathway, and thus effects

on cell-cell fusion must be validated using direct infection and/or fusion assays.

in vivo work using mice genetically resistant to infection showed that a hypercholesterolemic diet can make mice susceptible to MHV infection (an effect reversible with a normal diet) and that hepatocytes isolated from these hypercholesterolemic animals have increased virus adsorption and intracellular titer versus control animals (Braunwald *et al.*, 1991). To address the issue of whether or not this enhancement is due to changes in membrane fluidity, the genetically resistant A/J mice were fed one of two diets, either a hypercholesterolemic (HC) or a phosphatidylserine (PS) diet (Nonnenmacher *et al.*, 1994). The expected result is that the cholesterol would decrease membrane fluidity, while the PS would increase membrane fluidity. The HC diet increases hepatocyte susceptibility to MHV infection about 5 fold, while the PS diet has a significant dose-dependent inhibition. However, when fluorescence anisotropy measurements were performed on the cultured hepatocytes from these animals to characterize membrane fluidity, both the HC and PS diets produce an increase in fluidity, indicating that there is no correlation between fluidity and infection. These results are consistent with work by others showing that HC diets can be at least partially compensated by cellular homeostatic mechanisms, which tightly regulate the effects of increased dietary cholesterol on membrane fluidity (Larsen *et al.*, 1993). Taken together, these results suggest that cholesterol could play a role in both the initial MHV binding/fusion steps and the later stages of virus-induced cell-cell fusion. More work is necessary to fully understand the role of cholesterol in the lifecycle of MHV, but it is intriguing that dietary components may be able to modulate the genetically-determined susceptibility to virus infection.

6.2.3. Ebola Virus

Ebola virus is a member of the Filoviridae, filamentous enveloped RNA viruses that can cause severe hemorrhagic disease in humans. Similar to the work described earlier for HIV, recent work has focused on liposome fusion induced by the putative fusion peptide of the Ebola virus glycoprotein (Ruiz-Arguello *et al.*, 1998). The ability of synthetic peptides corresponding in sequence to the putative fusion peptide to induce fusion of liposomes is strictly dependent on the presence of Ca^{2+} in the reaction and phosphatidylinositol (PI) in the vesicle membranes. In fact, the best fusion rate and efficiency are achieved using large unilamellar vesicles with a lipid composition approximating that of the hepatocyte plasma membrane (PC : PE : Chol. : PI 2 : 1 : 1 : 0.5). The effect of PI is at least partially mediated by the initial interaction of the peptide and liposomes, but appears not to

be due only to the charge of the lipid because other anionic lipids (phosphatidylglycerol or phosphatidic acid) do not substitute. Additionally, PI from various animal and plant origins is interchangeable, suggesting that the acyl chain composition is unimportant. While these data suggest that PI may play a specific role in Ebola spike protein-mediated fusion, the previously mentioned caveats of work with isolated virus peptides apply here as well. Further *in vitro* and *in vivo* work will be needed to define the importance of PI in the context of Ebola virus fusion and infection.

6.2.4. African Swine Fever Virus

African swine fever virus (ASFV) is a unique animal DNA virus of an unnamed family. It has a complex structure with an overall icosahedral shape, formed by a DNA-containing viral core which is successively wrapped by two inner lipid bilayers derived from collapsed ER cisterna, a capsid protein with a hexagonal lattice structure, and an outer lipid membrane derived from the plasma membrane during virus budding (Andres *et al.*, 1997; Carrascosa *et al.*, 1984). ASFV is thought to infect cells via receptor-mediated endocytosis and low pH-triggered membrane fusion (Valdeira and Geraldes, 1985; Alcamí *et al.*, 1989).

A recent report used several approaches to address the role of cholesterol in ASFV infection (Bernardes *et al.*, 1998). When cellular cholesterol synthesis is inhibited by cerulenin, W-7 and miconazole, the production of progeny ASFV is decreased by ~50%. When the cellular cholesterol level is decreased to ~50% of that in control cells by culturing Vero cells in delipidated serum, similar kinetics of endocytic uptake are observed for ASFV, while the kinetics of virus penetration to the cytoplasm are significantly slowed. Low pH-triggered fusion of ASFV with the plasma membrane of cholesterol-depleted cells is inhibited compared to fusion with control cells. In addition, similar to results previously published for SFV (Phalen and Kielian, 1991), ASFV fusion with the plasma membrane is inhibited by cholesterol oxidase pretreatment of the target cells. Since this enzyme is known to oxidize the sterol 3 β -hydroxyl group, this result suggests that, similar to SFV, the sterol 3 β -hydroxyl group may be important in the ASFV cholesterol requirement. The role of sphingolipid in ASFV infection and fusion has not been addressed. These experiments with ASFV, while provocative, are complicated by our limited ability to deplete cholesterol in mammalian cell lines and the potential wide-ranging side effects of such sterol depletion (Nes and McKean, 1977a). It will be important to compare the cholesterol-dependence of ASFV fusion and infection in parallel with that of a virus known to be cholesterol independent, such as VSV, to control for non-specific effects of inhibitors and depletion. It will also be important to

characterize the cholesterol-dependence of ASFV fusion with liposomes of defined and physiological lipid composition.

6.2.5. Sendai Virus

Sendai virus is a member of the paramyxovirus family that fuses with host cell plasma membranes at neutral pH upon binding to its receptor, sialic acid (see (Lamb, 1993; Baker *et al.*, 1999) for review). It has two spike glycoproteins, the fusion protein (F) that contains the putative fusion peptide, and the hemagglutinin-neuraminidase protein (HN) that mediates receptor binding and may potentiate the fusion reaction. Several early studies suggested that cholesterol is required for Sendai virus fusion. By measuring Sendai virus fusion *in vitro* with purified liposomes, Hsu and Choppin (Hsu *et al.*, 1983) demonstrated that the presence of cholesterol in the liposomal membrane is necessary for efficient fusion, and that the optimal concentration of cholesterol is 0.3–0.4 mole fraction, similar to the optimal concentration for SFV-liposome fusion (White and Helenius, 1980). Kundrot and MacDonald studied liposome lysis in response to Sendai virus and mild hypo-osmotic stress, a reaction presumably reflective of virus-membrane fusion, and found that cholesterol is required (Kundrot *et al.*, 1983). Both of these liposome studies were performed using PC : cholesterol liposomes. Fusion of Sendai with a more complex biological membrane also appeared to be cholesterol-dependent, as assayed by Sendai fusion with *Mycoplasma* membranes containing varying amounts of sterol (Citovsky *et al.*, 1988). Asano and Asano tested the ability of purified F protein to bind radioactive cholesterol *in vitro*, and demonstrated that 5–14% of intact F protein binds cholesterol in a filtration assay (Asano and Asano, 1988). Reduced binding is observed for F protein in which the N-terminal fusion peptide is removed by thermolysin digestion, suggesting that the fusion peptide is involved in cholesterol binding. Sterol binding appeared to require the A/B ring trans structure of cholesterol, rather than the 3 β -hydroxyl group. It is important to note, however, that the purified F protein used for binding assays may not maintain its native conformation, and that the hydrophobic F protein transmembrane domain is present in the F protein preparations used. In addition, the binding assay may be oversimplified and does not take into account potential effects of the virus HN protein, receptor binding which is the presumed fusion trigger, or the normal target membrane lipid bilayer structure.

A cholesterol requirement has not been universally observed for Sendai virus fusion. Klappe *et al.* showed that Sendai virus could fuse with liposomes solely consisting of acidic phospholipids, particularly cardiolipin (Klappe *et al.*, 1986). Notably, however, virus fusion with cardiolipin-

containing liposomes is prone to aberrant effects from the charge and nature of this lipid (Stegmann *et al.*, 1989b). Cholesterol-independent fusion was also observed by Cheetham and Epan, using lipid-labeled Sendai virus with cholesterol-free liposomes containing PE and ganglioside (Cheetham *et al.*, 1990; Cheetham *et al.*, 1994). It is possible that the difference between PE and PC bilayer structure explains the differing results between this and the above liposome experiments. The incorporation of cholesterol sulfate into such PE liposomes was found to potentially inhibit both the rate and extent of Sendai virus-mediated fusion (Cheetham *et al.*, 1990; Cheetham *et al.*, 1994). Such inhibition is also observed for human erythrocyte ghost target membranes that are pre-incubated with cholesterol sulfate. The mechanism of inhibition is not clear, but is presumed to be an effect of cholesterol sulfate on membrane stability and physical properties.

Clearly, conflicting data exist on the role of cholesterol in Sendai virus-induced fusion. Further systematic studies using physiological lipid mixtures and sterol-depleted biological membranes will hopefully resolve these issues.

6.2.6. Role of Cholesterol in Transport of Influenza Hemagglutinin

Influenza virus is a member of the orthomyxovirus family whose fusion reaction and hemagglutinin (HA) fusion protein have been exceptionally well-characterized (Wiley and Skehel, 1987; White, 1992; Hughson, 1995; Carr and Kim, 1993; Bullough *et al.*, 1994). Influenza virus infects cells via the endocytic pathway where the low pH in endosomes triggers fusogenic conformational changes in HA, leading to the formation of an extended coiled-coil that mediates viral membrane fusion with endosomal membranes. Fusion studies with liposomes excluded a critical role of cholesterol, sphingolipids or other specific lipids in influenza virus fusion (White *et al.*, 1982; Stegmann and Helenius, 1993). However, substantial evidence suggests a role for cholesterol and sphingolipids in the virus exit pathway.

Influenza virus buds from the plasma membrane of infected cells, and HA transport and budding occur selectively at the apical domain of polarized epithelial cells (Ikonen and Simons, 1998). In either polarized or non-polarized cells, HA associates selectively with cholesterol and sphingolipid-enriched membrane domains termed "raft" domains, which are characterized by their resistance to extraction by Triton X-100 at 4°C (Brown and Rose, 1992). HA association involves the protein's transmembrane domain since replacement with a foreign transmembrane domain or point mutations in this region abolish HA-raft association (Scheiffele *et al.*, 1997). Depletion of cellular cholesterol results in dissociation of HA from raft domains, partial HA missorting to the basolateral domain, and a slower

transport rate of HA to the plasma membrane in both polarized and non-polarized cells (Scheiffele *et al.*, 1997; Keller and Simons, 1998). Analysis of purified fowl plague virus (FPV), an avian influenza virus, demonstrated that FPV particles contain a high percentage of detergent-resistant complexes enriched in cholesterol and sphingolipids (Scheiffele *et al.*, 1999). In contrast, purified VSV and SFV, both basolaterally-targeted viruses, were not similarly enriched in such complexes (Scheiffele *et al.*, 1999). These results suggest that influenza virus may select specialized membrane domains for both HA transport and virus budding, and that these domains may also be important in the virus's apical targeting pathway. Further studies will be necessary to demonstrate the importance of raft domains *in vivo* and to examine the effect of cholesterol or sphingolipid depletion on influenza virus budding. It is also important to note that transport to the apical domain most likely involves other mechanisms besides lipid rafts (Weimbs *et al.*, 1997; Brown and London, 1998). Although SFV budding from the plasma membrane is cholesterol-dependent, the fact that the SFV spike protein does not preferentially associate with lipid raft domains (Scheiffele *et al.*, 1999) suggests that influenza virus and SFV use different budding mechanisms, each of which may involve cholesterol.

6.3. Lipid Stalk Intermediates in Membrane Fusion Reactions

The previous discussion centers on the functions of various viral or bacterial proteins and the different ways in which they may interact with cholesterol or other lipids. We have focused on fusion proteins that may have specific interactions with particular lipids during fusion. In addition to these specialized lipid-requiring mechanisms, membrane fusion reactions may use a common, lipid-dependent structural intermediate during the actual fusion process. This has been termed the "stalk intermediate", referring to a strongly bent structure shaped like a stalk that mediates a local contact between the two lipid bilayers which are to fuse (Melikyan and Chernomordik, 1997; Chernomordik and Zimmerberg, 1995). Such an intermediate would involve primarily the lipids of the two fusing bilayers and would be similar regardless of the fusion proteins involved or the type of membrane fusion event under study (viral fusion, exocytosis, or intercellular fusion). The structure of this bent intermediate is strongly influenced by the types of lipids present in the membranes, and the respective geometries they impose on the membranes. The involvement of this stalk intermediate has been characterized primarily by observing the effects on fusion of lipids that favor a strongly bent stalk structure versus those that oppose it. (reviewed in Melikyan and Chernomordik, 1997; Chernomordik and Zimmerberg, 1995). For example, fusion is inhibited by lysophospholipids and

promoted by cis-unsaturated fatty acids. Available experimental and theoretical work suggests that a “stalk” fusion intermediate may be required in all fusion events (Kozlov and Markin, 1983; Siegel, 1993; Basanez *et al.*, 1998). The fusion proteins of viruses or cells could impose additional restraints on stalk formation or the subsequent fusion pore, but may be most important in the process of initiating lipid-lipid contact of the bilayers prior to formation of a stalk intermediate. While the role and importance of a lipidic stalk intermediate are currently an evolving area of investigation in the membrane fusion field, at the least these studies point out the potential importance of the effects of various lipids on the structural intermediates of bilayer fusion. It is possible that structural effects of differing lipid composition may alone account for some of the discrepancies reported for various virus-cell or virus-liposome experiments. The potential effect of lipid composition on bilayer structure and stalk formation should certainly be taken into account when trying to identify or ascribe a role for any particular lipids in viral or cellular fusion events.

6.4. Cellular Fusion Proteins

To date, viral spike proteins are the best understood fusion proteins and have significantly advanced both our general knowledge of fusion and our specialized understanding of the mechanisms by which changes in a protein's conformation lead to membrane fusion. All cells carry out fusion reactions as part of intracellular vesicular traffic during endocytosis and exocytosis (Hernandez *et al.*, 1996; Stegmann *et al.*, 1989a; Rothman and Warren, 1994). Many cells also fuse with other cells during a variety of developmental processes (Hernandez *et al.*, 1996; Stegmann *et al.*, 1989a; Podbilewicz and White, 1994). These cellular fusion events are numerous, highly specific, and tightly regulated. In many cases, the identity of the cellular fusion proteins is as yet unknown and the possible involvement of specific lipids is unexplored. Much of this remains to be addressed in future studies, but we will mention here several examples of proteins that may mediate cellular fusion events.

It is clear that most if not all intracellular vesicular fusion events involve proteins termed SNAREs which are found associated with either the vesicle (v-SNAREs) or target cell membrane (t-SNAREs) (Rothman and Warren, 1994; Bennett and Scheller, 1994). Recent structural information on v- and t-SNARE complexes is remarkable in that the structure of the SNARE complex has strong similarities to the extended α -helical coiled-coil believed to be operating in the fusogenic conformation of the influenza HA and other viral fusion proteins of this type (Sutton *et al.*, 1998). Thus these data suggest that viral and cellular fusion proteins may

act via similar mechanisms. While some studies indicate that the formation of the SNARE complex itself may drive membrane fusion (Weber *et al.*, 1998; Chen *et al.*, 1999), others suggest that the actual membrane fusion event does not require the SNARE complex (Ungermann *et al.*, 1998; Tahara *et al.*, 1998). This is an exciting and fast-moving area, and it will be important to determine how the SNARE proteins might be acting in membrane fusion, and whether such fusion events have any specific lipid requirements in the vesicle or target membranes.

During fertilization the sperm must first bind to the egg and then initiate a membrane fusion event so that the genetic material of the male can be deposited into the egg cytoplasm. The binding and fusion steps in this process have similarities to the other membrane fusion events discussed in this review. One of the proteins involved in sperm-egg fusion is the sperm surface protein fertilin, originally termed PH-30 (reviewed in Evans, 1999; Snell and White, 1996). This protein is a heterodimer, composed of a fertilin α and a fertilin β subunit, that may oligomerize into higher order structures. Both subunits are members of a family of proteins termed ADAMs (for A Disintegrin And Metalloprotease), and including other proteins implicated in fusion events, such as meltrin, which plays a role in myoblast fusion (Yagami-Hiromasa *et al.*, 1995). The fertilin α subunit contains a short stretch of residues (89–111) with similarities to and some homology to viral fusion peptides (Blobel *et al.*, 1992). Several studies with synthetic peptides corresponding to this putative fertilin fusion peptide have demonstrated its binding to liposomes and ability to fuse membranes under certain conditions (Martin *et al.*, 1998; Muga *et al.*, 1994; Niidome *et al.*, 1997; Martin and Ruyschaert, 1997). No specific lipid requirements have been observed to date. A number of genetic and biochemical experiments argue for the importance of ADAMs in both sperm-egg and myoblast fusion (Yagami-Hiromasa *et al.*, 1995; Wolfsberg and White, 1996; Cho *et al.*, 1998), and suggest that these interesting molecules play a significant role although it is too soon to conclude that they are themselves acting as fusion proteins.

A second protein implicated in sperm-egg fusion is bindin, a membrane-associated acrosomal sperm protein in sea urchin (Ulrich *et al.*, 1998; Glabe, 1985a; Glabe, 1985b). Bindin has been shown to bind to and fuse liposomes, and has particular affinity for gel phase membrane domains. In particular, bindin-mediated liposome fusion occurs rapidly when sphingomyelin/cholesterol liposomes are used (Glabe, 1985b). A minimal "fusion peptide" region of bindin has been characterized and shown to have fusion activity with sphingomyelin/cholesterol liposomes (Ulrich *et al.*, 1998). Such peptide and isolated protein studies require more extensive verification and experimentation in order to meaningfully interpret the importance of

bindin in fusion, but the lipid dependence of this reaction adds to the potential interest in bindin as a candidate fusion protein.

7. FUTURE DIRECTIONS

It is clear that while numerous lines of evidence point to potential roles for lipids in the function of fusion proteins and membrane budding reactions, in many cases this evidence remains at a very early stage, and much remains to be done to provide convincing proof that protein-lipid interactions are critical. In order to determine a definite role for lipid in a biological fusion reaction, it is clearly important to test the effect of lipid *in vitro* using a system as close to the biological fusion reaction as possible. This includes using the native virus fusion protein and the natural trigger for fusion, whether it be low pH or receptor interactions. The *in vivo* role of lipid in fusion or exit should be tested by using a lipid-modified cell system if this is possible, carefully controlling for the non-specific effects of lipid modification by evaluating cellular functions and by parallel experiments using viruses or proteins that are not lipid dependent. In particular, the interpretation of cell-cell fusion experiments must be carefully done, as this morphological assay can be affected secondarily by substances that do not affect the fusion step per se. Given that complete depletion of a specific lipid may be difficult or impossible, it may be necessary to correlate the amount of lipid depletion with the amount of inhibition observed. It is also important in all of these experimental approaches to determine the dependence of a lipid effect on the structural features of the lipid, and to evaluate any lipid effect in the context of membrane fluidity, charge, and bilayer stalk formation. While no experimental system is perfect and it is important that the role of lipids in fusion and exit be evaluated in spite of such shortcomings, at the least such guidelines provide a critical framework for the interpretation of experimental results.

Within the alphaviruses, both fusion and virus exit have well-defined and substantial lipid requirements. The *in vivo* role of sphingolipid has not been addressed, and it will be important to apply future advances in our ability to modify cellular sphingolipids to test the importance of sphingolipid *in vivo*. The challenge in the alphavirus field is now to determine the molecular mechanisms by which cholesterol and sphingolipids interact with the viral proteins to exert their effects on the virus life cycle. We need to determine how these two lipids act to promote fusogenic conformational changes, and the individual and synergistic roles of each lipid in this process. How might the spike protein interact with the membrane to “sense” the presence of cholesterol and/or sphingolipid, even prior to the stable inser-

tion of the virus fusion peptide? A key question is the role of specific lipids and target membrane bilayer in the formation of the EI homotrimer structure. As part of this, it will be important to determine the potential structural differences in the homotrimer formed in the presence of a fusion-permissive membrane versus a fusion-inactive membrane. Increased structural information on the monomeric and trimeric forms of the EI fusion protein and the availability of virus mutants altered in their lipid requirements should point the way to understanding the sites and functions of lipid-protein interaction. Our understanding of the role of cholesterol in virus exit should become more mechanistic with advances in our knowledge of virus and spike protein structures, and more molecular analysis of the virus exit pathway. In addition, it is important for this and other virus systems to understand the relevance of such lipid requirements to infection of various types of cells in tissue culture, and to viral infection and pathogenesis in animal hosts. Ultimately, a better understanding of the function of particular lipids in virus lifecycles should enable us to design specific strategies to interfere with their function and thus limit virus replication.

ACKNOWLEDGMENTS. We thank Dr. Sallie Glomb-Reinmund for preparing Figure 1 and Dr. Marianne Marquardt for preparing Figure 2. The work from our laboratory described in this review was supported by grants to M.K. from the National Institutes of Public Health (GM 52929 and GM 57454), the American Cancer Society (RPG-93-013-07-MBC), the Hirsch Charitable Trust, by the Jack K. and Helen B. Lazar fellowship in Cell Biology, and by Cancer Center Core Support Grant NIH/NCI P30-CA13330. D.L.G. was supported by an MSTP training grant from the National Institutes of Public Health (T32 GM07288).

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Chapter 12

Fusion Mediated by the HIV-1 Envelope Protein

Carrie M. McManus and Robert W. Doms

1. INTRODUCTION

Retroviruses are a family of enveloped RNA viruses that utilize reverse transcriptase to convert viral RNA into linear double-stranded DNA. All retroviruses contain several key structural elements. These include *gag*, which encodes the internal structural proteins, *pol*, which encodes for the reverse transcriptase and integrase, and *env* which encodes the viral envelope (Env) protein (reviewed in (Trono, 1994; Turner and Summers, 1999)). Human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS), is a particularly well studied retrovirus and is the subject of this chapter. There are numerous steps involved in the infection process, beginning with receptor binding and membrane fusion. In the case of HIV, the viral Env protein mediates both receptor binding as well as fusion between the viral and cellular membranes. Recent advances in identifying receptors involved in virus entry as well as the elucidation of key structural elements in Env have greatly increased our under-

CARRIE M. MCMANUS and ROBERT W. DOMS Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, 19104.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

standing of HIV entry, cellular tropism, and pathogenesis. In addition, studies on viral entry have identified attractive new cellular and viral targets for antiretroviral agents. In this chapter we will discuss the current understanding of the viral and cellular components necessary for the viral entry process and what remains to be understood.

2. VIRAL COMPONENTS OF FUSION

2.1. Env

The Env protein is responsible for binding virus to the cell surface by interactions with CD4 and, through a series of conformational changes, a coreceptor. These interactions in turn catalyze Env-mediated fusion between the viral and cellular membranes (Wyatt and Sodroski, 1998). The HIV-1 Env glycoprotein (gp) is synthesized as a precursor protein of approximately 160kD termed gp160, which assembles into a trimeric structure in the endoplasmic reticulum (Earl *et al.*, 1991). Following transport to the Golgi apparatus, the gp160 is cleaved by an as yet unidentified cellular protease into surface (SU) and transmembrane (TM) subunits termed gp120 and gp41, respectively. The gp120 subunit binds to cell surface receptors and, as a result, largely governs viral tropism at the level of entry while gp41 promotes fusion of the viral and cellular membranes resulting in the release of the viral genome into the cell. Topologically, the Env protein is similar to many other viral membrane fusion proteins, such as the influenza hemagglutinin (HA), which is likewise synthesized as a type I membrane protein that is posttranslationally cleaved into two subunits (Hernandez *et al.*, 1996). As for HA, cleavage of the HIV gp160 precursor into SU and TM subunits is required to render the protein competent to mediate membrane fusion (McCune *et al.*, 1988). Cleavage liberates the N-terminal domain of gp41 that constitutes the “fusion peptide” (discussed below) that is essential for membrane fusion.

2.1.1. Gp120

The gp120 subunit is a complex molecule that is extensively glycosylated and contains 9 disulphide bonds (Leonard *et al.*, 1990). In addition to playing an important role in protein folding, these carbohydrates likely enhance the stability of Env by providing protection from proteolytic enzymes and reducing immunogenicity (Wyatt and Sodroski, 1998). Comparison of multiple Env sequences shows that gp120 is composed of

variable and more highly conserved domains. The variable domains are typically composed of disulfide bonded-loops that project from the surface of the molecule. While neutralizing antibodies can be directed to these regions, their variable nature makes neutralization of divergent virus strains difficult (reviewed in (Moore and Ho, 1995)). The recently solved structure of a gp120 core fragment has shown that the gp120 core is composed of an inner domain connected to an outer domain by a bridging sheet (Kwong *et al.*, 1998; Wyatt and Sodroski, 1998). The inner domain likely faces gp41 while the heavily glycosylated outer domain is exposed on the surface. CD4 binds in a pocket on gp120 and has numerous contact sites at the interface of the outer domain and the bridging sheet. The bridging sheet contains the V1/V2 stem and part of the fourth conserved region. The structure also revealed a highly conserved coreceptor binding site that is largely located in the bridging sheet between the bases of the V3 loop and the V1/V2 region ((Rizzuto *et al.*, 1998) and Figure 1). Amino acid substitutions within this region adversely affect gp120-CCR5 interactions (Rizzuto *et al.*, 1998). The role of this domain as well as the variable regions in coreceptor interactions is discussed below.

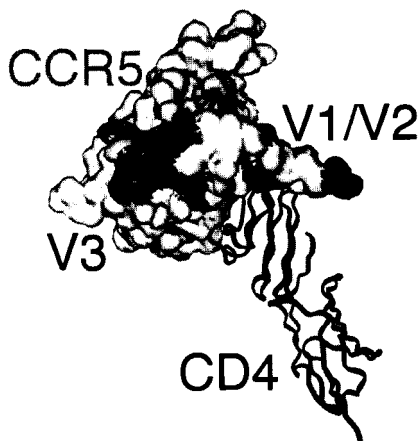


FIGURE 1. Space filling model of HIV gp120 core fragment bound to CD4, shown as a ribbon diagram (Kwong *et al.*, 1998). Depicted are those regions important for CCR5 binding (Rizzuto *et al.*, 1998) as well as the V1/V2 stem and the base of the V3 loop, residues of which are determinants for coreceptor use. The variable loops themselves as well as other portions of gp120 were not included in the core fragment.

2.1.2. Gp41

Gp41 is a transmembrane protein of approximately 330 amino acids with a hydrophobic, glycine and alanine-rich fusion peptide located at the gp41 N-terminus. Thus, cleavage of gp160 to form gp120 and gp41 subunits also generates the N-terminus of the fusion peptide. Like other viral fusion peptides, the HIV-1 Env fusion peptide is hydrophobic (Hernandez *et al.*, 1996). In addition to being hydrophobic, the fusion peptide is highly conserved among different virus strains. Mutations within the fusion peptide have been shown to adversely affect membrane fusion without affecting Env processing, cleavage, or receptor binding (Delahunty *et al.*, 1996; Freed *et al.*, 1992; Freed *et al.*, 1990; Kowalski *et al.*, 1987). Some mutations dominantly interfere with membrane fusion (Freed *et al.*, 1992), suggesting that a higher-order structure is involved in the fusion reaction. By analogy with the far better characterized influenza HA, it is thought that the HIV-1 fusion peptide becomes exposed and inserts into the target membrane, thus making Env an integral component of two membranes. However, this has not been demonstrated experimentally. With the discovery of the coreceptors, it should be possible to examine the conditions under which the fusion peptide becomes exposed and to better characterize its interaction with the target membrane.

While the structure of native gp41 has not been elucidated, inspection of the gp41 sequence reveals the presence of two leucine zipper motifs (Delwart *et al.*, 1990). Originally thought to play a role in Env oligomerization, these domains are now known to play important roles in the membrane fusion process (Chen, 1994; Chen *et al.*, 1993; Dubay *et al.*, 1992; Wild *et al.*, 1994). In fact, many other viral fusion proteins likewise contain two helical domains in their transmembrane domain subunits, including influenza HA (Chan *et al.*, 1997). During the conformational changes that lead to membrane fusion, these helical domains are thought to interact with one another, forming a packed helix bundle and repositioning the fusion peptide in the direction of the target membrane. In fact, crystallization of peptides based on this region shows that the N-terminal peptides form a trimeric coiled-coil while the C-terminal peptides pack anti-parallel into hydrophobic grooves on the surface of the coiled-coil (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). Several synthetic peptides which model this region potently inhibit Env-mediated fusion and virus infection *in vitro* (Wild *et al.*, 1994; Wild *et al.*, 1995; Wild *et al.*, 1992; Wild *et al.*, 1994) and have led to dramatic decreases in viral load *in vitro* (Kilby *et al.*, 1998), likely by complexing to either the N- or C-terminal motifs of gp41 and interfering with the formation of the 6-helix "hairpin" (Figure 2).

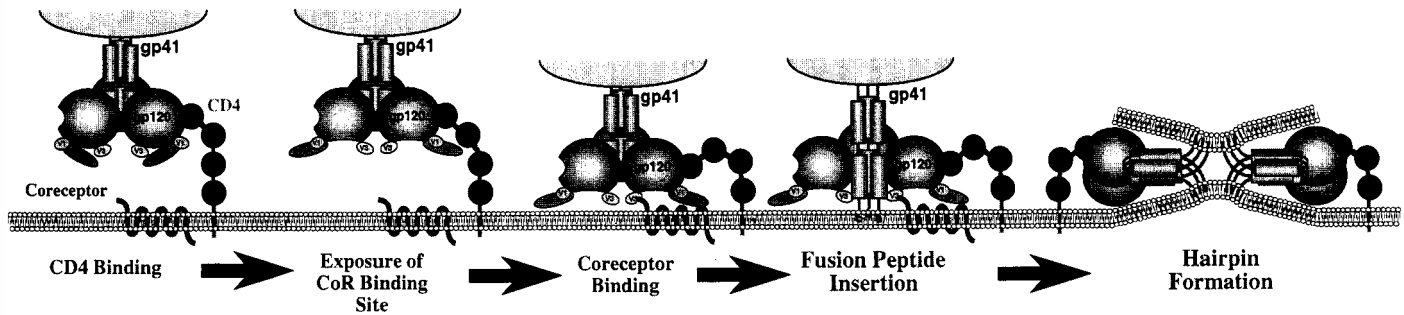


FIGURE 2. Model for HIV-1 Entry. HIV-1 entry requires several sequential steps to allow viral fusion to occur. Step 1 is CD4 binding to the viral envelope protein. This results in exposure of the coreceptor binding site allowing binding of the CD4-Env complex to the coreceptor. The interaction of this complex results in exposure of the fusion peptide, hairpin formation, and lipid mixing allowing HIV entry into the cell.

3. CELLULAR COMPONENTS OF FUSION

3.1. CD4

CD4 was identified as the major cellular receptor for HIV shortly after the discovery of HIV itself. This was determined by the fact that HIV infects CDC-positive cells almost exclusively, antibodies to CD4 can block virus infection, introduction of CD4 into otherwise CD4-negative human cells typically renders them permissive to infection by at least some HIV strains, and by the fact that gp120 binds directly to CD4 (Dalglish *et al.*, 1984; Klatzman *et al.*, 1984; Maddon *et al.*, 1986; McDougal *et al.*, 1986; McDougal *et al.*, 1985). Expression of CD4 on T-cell subsets, monocytes, macrophages, dendritic cells, and microglia is largely responsible for the selective infection of these cell types. CD4 is a 55kD glycoprotein that is a member of the immunoglobulin superfamily. It has four extracellular immunoglobulin-like domains (D1-D4) as well as a membrane spanning region and a short cytoplasmic tail (Ryu *et al.*, 1991; Wang *et al.*, 1990). The high-affinity binding site for gp120 is composed largely of a stretch of 20 amino acids in the D1 region. Mutagenesis studies indicated that residue Phe43 is particularly important for gp120 binding (Ryu *et al.*, 1994). In fact, the recently solved structure of gp120 shows that Phe43 fits into a conserved, hydrophobic pocket in the gp120 subunit, making this region an attractive target for small molecule inhibitors (Kwong *et al.*, 1998). While binding to CD4 triggers conformational changes in Env, these are not sufficient to result in membrane fusion. Rather, these changes enable Env to make subsequent interactions with an appropriate coreceptor. Coreceptor binding is then thought to trigger the final conformational changes in Env that lead to membrane fusion (Figure 2).

3.2. The Major HIV-1 Coreceptors

Following the discovery of CD4 as the HIV receptor it was determined that not all cells that are CD4 positive can support HIV entry. Two major lines of experimental work illustrated this point. First, expression of human CD4 in non-human cells fails to make them permissive for Env-mediated membrane fusion and hence, virus infection (Ashorn *et al.*, 1990; Chesebro *et al.*, 1990; Clapham *et al.*, 1991; Maddon *et al.*, 1986). Second, many CD4-positive human cell lines support infection by only particular subsets of HIV-1 strains (Cheng-Mayer *et al.*, 1988; Fauci, 1996; Fenyö *et al.*, 1988). In general, HIV-1 strains can be divided into groups depending on their tropism for particular types of CD4-positive cells. Macrophage-tropic (M-tropic) viruses enter macrophages and primary T-cells but not T-cell lines,

while T-tropic viruses enter T-cell lines and primary T-cells, but often fail to enter macrophages (Schuitemaker *et al.*, 1992). This differential tropism is clinically important, since M-tropic virus strains are largely responsible for virus transmission and are the most common type of virus isolated from asymptomatic individuals (Roos *et al.*, 1992; Schuitemaker *et al.*, 1992; Zhu *et al.*, 1993), while T-tropic viruses tend to emerge (as a consequence of mutations in Env) after a period of years (Tersmette *et al.*, 1989; Tersmette *et al.*, 1989). Emergence of T-tropic virus strains is associated with accelerated progression to AIDS (Connor *et al.*, 1993; Schuitemaker *et al.*, 1992; Tersmette *et al.*, 1989; Tersmette *et al.*, 1989). Taken together, these observations indicate that HIV requires, in addition to CD4, a coreceptor to infect cells, and that M-tropic and T-tropic virus strains might utilize different coreceptors. Since the block to virus infection in the absence of a coreceptor is at the level of membrane fusion, coreceptors are likely to serve as the "trigger" that enables Env to undergo the conformational changes needed to mediate membrane fusion.

After a number of false leads, the first bona-fide HIV coreceptor was identified by Berger and colleagues in 1996, and was initially called Fusin for its ability to facilitate viral fusion and entry by T-tropic (but not M-tropic) virus strains (Feng *et al.*, 1996). Fusin, a 46kD transmembrane glycoprotein, was later found to be a member of the chemokine receptor family of G-protein coupled seven transmembrane spanning proteins (Bleul *et al.*, 1996; Oberlin *et al.*, 1996). The chemokine receptors serve as receptors for chemoattractant cytokines which are involved in leukocyte transmigration and inflammation (reviewed in Rollins, 1997). Chemokines are named based on the position of their N-terminal cysteines with or without intervening amino acids and this corresponds to the receptor names which include the C-C, C-X-C, C, and C-X 3-C receptors. The receptor termed Fusin was then named CXCR4 as it is a receptor for the CXC chemokine stromal-derived factor-1 (SDF-1). Numerous studies have shown that while CXCR4 is a coreceptor for T-tropic virus strains, it fails to support infection by M-tropic viruses (Berson *et al.*, 1996; Feng *et al.*, 1996). Introduction of CXCR4 and CD4 into almost any cell type renders the cell permissive to T-tropic but not M-tropic Env-mediated membrane fusion.

CXCR4 is expressed on a variety of cells including monocytes, neutrophils and lymphocytes and a variety of T cell lines (Bleul *et al.*, 1997; Forster *et al.*, 1998; Hesselgesser *et al.*, 1998; Ostrowski *et al.*, 1998). Compared to CCR.5, CXCR4 is expressed preferentially on the CD45RA⁺ subset of activated memory T-cells, while CCR.5 is expressed preferentially on CD45RO⁺ T-cells (Bleul *et al.*, 1997). Macrophages also express abundant levels of CXCR4 (Lee *et al.*, 1999), though there is considerable donor

variability and the method used to culture macrophages can strongly affect receptor expression (Lee *et al.*, 1999; Yi *et al.*, 1998). Human microglia, neurons, astrocytes and endothelial cells have also been reported to express CXCR4 (Albright *et al.*, 1999; Hesselgesser *et al.*, 1997; Lavi *et al.*, 1997; Vallat *et al.*, 1998; Zhang *et al.*, 1998). Recently, CXCR4 and SDF-1 knock-out mice have been generated, with homozygous disruption of these genes being lethal during development (Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Zou *et al.*, 1998). Whether CXCR4 function is critical for normal human health after development is not yet known but must be considered when developing antiviral drugs which may target this receptor.

The identification of CXCR4 as the major coreceptor for T-tropic HIV-1 strains suggested that the more prevalent M-tropic viruses would utilize a structurally related molecule. The discovery that the chemokines RANTES, MIP-1 α , and MIP-1 β could inhibit infection by M-tropic viruses (Cocchi *et al.*, 1995) indicated that the cognate receptor for these chemokines would be a likely candidate for the M-tropic virus coreceptor. Parmentier and colleagues cloned such a receptor, CCR5 (Samson *et al.*, 1996), and it was quickly shown to function as a coreceptor for M-tropic HIV-1 strains (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996). This led to a new nomenclature identifying viruses as either X4 (for CXCR4 utilizing) or R5 (for CCR5 utilizing) rather than M- or T-tropic (Berger *et al.*, 1998). Dual-tropic viruses that can utilize both coreceptors are termed R5X4. CCR5 has been found to be highly expressed on mucosal surfaces (Rottman *et al.*, 1997) as well as numerous cells such as monocytes, macrophages, peripheral blood derived dendritic cells, and T cells (particularly the Th1 type) (Lee *et al.*, 1999; Ostrowski *et al.*, 1998; Tuttle *et al.*, 1998; Zhang *et al.*, 1998). Introduction of CCR5 into most cell types in conjunction with CD4 renders them permissive for M-tropic (R5) Env-mediated membrane fusion.

3.2.1. The Importance of CCR5 and CXCR4 *In vitro* and *In vivo*

The studies described above provide compelling evidence that CCR5 and CXCR4 are the major HIV-1 coreceptors *in vitro*, and that their presence in conjunction with CD4 is essential for viral infectivity. A dramatic demonstration of the importance of CCR5 for virus infection *in vitro* was provided by the finding that approximately 1% of Caucasians are CCR5-negative due to a naturally occurring 32-base pair deletion that results in a truncated molecule that is not expressed at the cell surface (Liu *et al.*, 1996; Samson *et al.*, 1996). Individuals who lack CCR5 are highly resistant (though not absolutely immune) to HIV infection, while D32-heterozygotes exhibit delayed progression to AIDS (Dean *et al.*, 1996; Huang *et al.*, 1996;

Liu *et al.*, 1996; Michael *et al.*, 1997; Samson *et al.*, 1996). This finding helps explain why some individuals remain uninfected despite multiple exposures to virus, but begs the question as to why X4 virus strains fail to establish infections in naive hosts with such low efficiency. The importance of CXCR4 for virus infection *in vitro* is strongly supported by the facts that many HIV strains can efficiently use this receptor to infect cells (Doms and Moore, 1997), CXCR4 can support infection of primary T-cells and macrophages, and that CXCR4 is the only coreceptor shown thus far to support infection of CCR5-negative primary human cells by HIV-1 (Simmons *et al.*, 1998; Yi *et al.*, 1998; Zhang and Moore, 1999).

3.3. Alternative HIV-1 Coreceptors

While all HIV-1 strains studied to date use CCR5, CXCR4, or both as coreceptors, a large number of additional chemokine and related orphan receptors are capable of supporting infection by subsets of HIV-1 strains *in vitro*. These include CCR2, CCR3, CCR8, CX3CR1, GPR1, GPR15, STRL33, ChemR23, and APJ (Choe *et al.*, 1996; Deng *et al.*, 1997; Doranz *et al.*, 1996; Edinger *et al.*, 1998; Farzan *et al.*, 1997; Horuk *et al.*, 1998; Liao *et al.*, 1997; Reeves *et al.*, 1997; Rucker *et al.*, 1997; Samson *et al.*, 1998). In general, these receptors support infection by relatively small numbers of virus strains, and typically do so less efficiently than CCR5 or CXCR4. Use of alternative coreceptors is more common by T-tropic than M-tropic virus strains, but does not strictly correlate with viral tropism. Potentially, use of alternative coreceptors could broaden host range by enabling viruses to infect additional types of CD4-positive cells. However, use of alternative receptors by HIV-1 to infect primary cells has not been demonstrated. The most illuminating experiments that address this point have tested the ability of diverse virus isolates to infect primary cells from individuals who lack CCR5. Thus far, infection of CCR5-negative primary human cells has only been shown to occur via CXCR4, arguing that the alternative receptors identified to date do not support infection of PBMC or macrophages (Simmons *et al.*, 1998; Yi *et al.*, 1998; Zhang and Moore, 1999). Interestingly, some SIV strains can infect CCR5-negative human cells in a CXCR4-independent manner, indicating that one or more alternative coreceptors are expressed at appropriate levels to support infection by a primate lentivirus (Chen *et al.*, 1997). To fully evaluate the potential *in vitro* significance of alternative coreceptors for HIV-1, it will be important to determine the numbers and types of viruses that can use any given receptor, the efficiency with which it is used, and determine where it is expressed and at what levels. The development of antibodies and small molecule inhibitors to these receptors will assist in these studies.

4. ENVELOPE-RECEPTOR INTERACTIONS

As discussed above, much progress has been made in characterizing the cellular and viral components involved in HIV Env-mediated membrane fusion. A current working model (see Figure 2) is that binding of CD4 triggers conformational changes in Env that enable it to interact with an appropriate coreceptor. Exposure or induction of the conserved coreceptor binding site in gp120 appears to be a component of these conformational alterations. It is of interest to note that gp120 retains the ability to interact with coreceptor long after CD4 binding (Doranz *et al.*, submitted). Thus, CD4 binding triggers a stable conformational change in Env, perhaps accounting for the ability of HIV to use very low levels of coreceptor to infect cells (Platt *et al.*, 1997; Platt *et al.*, 1998). Coreceptor binding, in turn, may serve as the trigger event that leads to conformational changes in Env, particularly in the gp41 subunit, that lead to membrane fusion. These changes are discussed below. This working model implies direct interactions between Env and a coreceptor, and this in fact has been demonstrated (Lapham *et al.*, 1996). The most commonly used assay to measure Env-coreceptor interactions has been to iodinate gp120 and determine whether it binds to cells expressing the desired coreceptor (Hesseltger *et al.*, 1997; Trkola *et al.*, 1996; Wu *et al.*, 1996). gp120 binding to CCR5 is dependent upon the presence of soluble CD4, occurs rapidly, and with an affinity of approximately 4nM for HIV-1 JRFL (Doranz *et al.*, submitted). Binding to CCR5 can be blocked by chemokines, by some antibodies to CCR5, and by V3-loop antibodies (Doranz *et al.*, submitted; Hill *et al.*, 1997; Lee *et al.*, 1999; Trkola *et al.*, 1996; Wu *et al.*, 1996). In addition, gp120 binding is very sensitive to receptor alterations (Baik *et al.*, submitted). Multiple mutations in CCR5, including many that do not significantly affect coreceptor function, prevent measurable gp120 binding. Thus, gp120 binding does not accurately predict coreceptor activity (Baik *et al.*, submitted). This is particularly true for the dual-tropic HIV-1 Env 89.6. HIV-1 89.6 efficiently uses CCR5 and CXCR4 to infect cells, and can also use a number of alternative coreceptors, including CCR2, CCR3, CCR8, STRL33, and APJ. However, 89.6 gp120 does not bind to any of these receptors at detectable levels, suggesting that the multi-tropic nature of this protein comes at the cost of not binding to any coreceptor with high affinity (Baik *et al.*, submitted).

The discrepancies between gp120 binding and coreceptor activity may have more to do with the assay systems employed than with how HIV uses coreceptors to infect cells. In the case of HIV-1 89.6 Env, it clearly must be competent to interact with a large number of coreceptors even though its gp120 subunit does not bind to any of these molecules at detectable levels.

There are several reasons that may account for this. First, Env on the surface of virions is trimeric, and may be capable of interacting with multiple coreceptors. By contrast, the cell surface binding assays most commonly employed use monomeric gp120. Second, after binding to a coreceptor, conformational changes may rapidly occur in Env that lead to membrane fusion, essentially making coreceptor binding an irreversible process. This clearly can not occur when monomeric gp120 is used. These findings underscore the importance of measuring both binding capabilities and functional properties of Env and receptor mutants.

4.1. Env Determinants of Coreceptor Use

Prior to the identification of the coreceptors it was shown that certain regions of Env were largely responsible for governing viral tropism at the level of entry. The V3 loop is the most important determinant of viral tropism, and introduction of a V3-loop derived from an M-tropic virus into a T-tropic Env is often sufficient to confer M-tropism (Chesebro *et al.*, 1991; DeJong *et al.*, 1992; Hwang *et al.*, 1991; Shioda *et al.*, 1992). The converse is also true. In addition to the V3-loop, the V1/V2 region also plays a role in governing viral tropism (Boyd *et al.*, 1993; Cao *et al.*, 1997; Cho *et al.*, 1998; Groenink *et al.*, 1993; Koito *et al.*, 1994). In some cases, changes in the V1/V2 region alone can alter tropism (Boyd *et al.*, 1993; Groenink *et al.*, 1993). With the discovery of the HIV coreceptors, it is now known that these changes are largely responsible for coreceptor choice. Changes in the V3 loop alone can frequently govern selection between CCR5 and CXCR4 (Bieniasz *et al.*, 1997; Cho *et al.*, 1998; Choe *et al.*, 1996; Cocchi *et al.*, 1996; Speck *et al.*, 1997). The V1/V2 region is also important for governing coreceptor choice, with use of alternative coreceptors perhaps being influenced more strongly by this region (Hoffman *et al.*, 1998; Ross and Cullen, 1998).

Demonstrations that the V3 loop and V1/V2 region largely govern coreceptor choice at first glance provides a simple explanation for how Env interacts with coreceptors. However, the variable nature of these domains raises a paradox: how can such variable regions be solely responsible for interactions between Env and CCR5 or CXCR4? The fact that so many divergent virus strains utilize CCR5 suggests that they must share some common structural motif that participates in coreceptor interactions. The structure of gp120 has provided at least a partial explanation, in that a highly conserved region of Env implicated in coreceptor binding has been identified (Rizzuto *et al.*, 1998). This domain, located largely in the bridging sheet between the bases of the V3 loop and V1/2 region, is extraordinarily well conserved (Figure 1). Mutations in this domain have been shown to affect binding of gp120 to CCR.5 (Rizzuto *et al.*, 1998). Whether this

domain also affects CXCR4 binding or interactions with alternative coreceptors is not known, but is likely due to the fact that this domain is largely conserved regardless of viral tropism. The respective roles of the variable regions and the conserved coreceptor binding site in CCR5 and CXCR4 interactions remain to be elucidated. Perhaps the variable regions make initial contact with the coreceptor, governing which coreceptor is used, while interactions between the conserved coreceptor binding site and other regions of the coreceptor trigger the conformational changes that lead to membrane fusion.

4.2. CCR5 Determinants

Numerous studies have examined the regions of CCR5 that are important for both coreceptor and chemokine receptor activity, with generally good agreement. Early studies using receptor chimeras demonstrated that all four extracellular domains of CCR5 are important for coreceptor activity, with the N-terminal domain and second extracellular loop (ECL) being the most important (Atchison *et al.*, 1996; Doranz *et al.*, 1997; Picard *et al.*, 1997; Rucker *et al.*, 1996). Further, these studies suggested that R5X4 virus strains are more sensitive to perturbations in the CCR5 N-terminus than are R5 viruses, and that there are differences in how HIV strains interact with CCR5 (Bieniasz *et al.*, 1997; Lu *et al.*, 1997; Rucker *et al.*, 1996). These observations have been confirmed by many other studies that have used both receptor chimeras as well as point mutants (Doranz *et al.*, 1997; Dragic *et al.*, 1998; Gwenaël *et al.*, 1998; Hill *et al.*, 1998; Picard *et al.*, 1997; Ross and Cullen, 1998). The N-terminal domain of CCR5 is clearly important for Env binding, and contains a tyrosine-rich region that is present in many alternative coreceptors (Farzan *et al.*, 1998; Martin *et al.*, 1997). Mutations in this domain adversely affect virus infection and can also block binding of gp120-sCD4 complexes to CCR5. Antibodies to the N-terminal domain of CCR5 also block gp120 binding, but are surprisingly ineffective at preventing virus infection (Lee *et al.*, 1999). Thus, the ability of an antibody to block gp120-CCR5 binding does not accurately predict its neutralization potential (Lee *et al.*, 1999; Siciliano *et al.*, 1999). This apparent discrepancy may be explained by the fact that other domains of CCR5 are also important for productive infection, with much attention being focused on ECL2. ECL2 is also important for chemokine binding (Samson *et al.*, 1997; Wu *et al.*, 1997), and may be involved in triggering conformational changes in Env that lead to membrane fusion (Edinger *et al.*, 1999). Antibodies to ECL2 often fail to block gp120 binding, but are typically more effective at blocking virus infection (Lee *et al.*, 1999; Siciliano *et al.*, 1999). A model to account for these findings is that while the N-terminal domain is important

for gp120 binding, interactions between Env and the ECLs may be involved in triggering the conformational changes that lead to membrane fusion. Indeed, some mutations in the CCR5 N-terminus that block gp120 binding are still compatible with significant coreceptor function, perhaps because in the context of virus infection CD4 provides a high affinity binding site for Env (Baik *et al.*, submitted; Edinger *et al.*, 1999).

4.3 CXCR4 Determinants

While CXCR4 has not been studied as extensively as CCR5, there is good agreement on the regions of this receptor that are most important for coreceptor and chemokine receptor activity. Through the use of chimeras and point mutants, it has been determined that the 1st and 2nd ECLS of CXCR4 are the most important domains for coreceptor function (Brelot *et al.*, 1999; Brelot *et al.*, 1997; Doranz *et al.*, 1999; Lu *et al.*, 1997; Picard *et al.*, 1997). However, chimeras containing only the CXCR4 ECL1 and ECL2 domains support infection less efficiently than the wild-type receptor, indicating that other domains can also play a role. Interestingly, some point mutations in the CXCR4 ECL2 domain enable this receptor to be used by R5 virus types (Wang *et al.*, 1998), again underscoring the importance of this domain for coreceptor activity and demonstrating that, as for Env, relatively subtle changes in receptor structure can have significant effects on viral tropism.

4.4. Conformational Changes Resulting from Receptor Interactions

Discovery of the HIV coreceptors should now make it possible to study the conformational changes that occur in Env during the course of membrane fusion. Binding to CD4 clearly results in conformational changes that either expose or induce the conserved coreceptor binding site. Coreceptor binding in turn leads to additional conformational changes, including exposure of the fusion peptide. With time, a helical hairpin is formed in the gp41 subunit as was discussed above. The time course of these conformational changes are not well understood, nor is it known if gp120 remains associated with the fusion complex or is shed. It has been shown that conformational changes occur within 1–4 minutes of receptor binding and are complete within 20 minutes which is prior to any evidence for lipid mixing (Dimitrov *et al.*, 1992; Jones *et al.*, 1998; Weiss *et al.*, 1996). This was shown by video microscopy and dye transfer assays. Fusion may necessitate the aggregation of gp41 trimers to form fusion pores because after fusion the fusion peptide and gp41 lie in the same membrane (Chan and Kim, 1998).

5. CD4-INDEPENDENT VIRUS INFECTION

An interesting variation of the HIV entry pathway is CD4-independent virus infection. Two HIV-1 isolates have been described that, as a consequence of *in vitro* passaging on CD4-negative cell lines, acquired the ability to infect cells in a CD4-independent, CXCR4-independent manner (Dumonceaux *et al.*, 1998; LaBranche *et al.*, submitted). The ability of one of these viruses, termed IIIBx, to use CXCR4 as a primary virus receptor is due in part to mutations that result in the constitutive, stable exposure of the conserved coreceptor binding site in gp120 (Hoffman *et al.*, 1999). Thus, gp120 derived from this virus binds directly to CXCR4 in the absence of CD4, and CD4-induced antibodies whose determinants largely overlap with the conserved coreceptor binding site bind efficiently to this CD4-independent Env. By contrast, binding of these antibodies to the parental, CD4-dependent IIIB Env occurs efficiently only after CD4 binding (Hoffman *et al.*, 1999). In addition to mutations that result in the exposure of the coreceptor binding site, alterations in gp41 also play a role in the CD4-independent phenotype (LaBranche *et al.*, submitted). However, mutations in the V3-loop do not contribute to CD4-independence. In fact, substitution of the IIIBx V3-loop with that from a virus that uses CCR5 results in an Env protein that mediates CDC-independent, CCR5-dependent virus infection (Hoffman *et al.*, 1999; LaBranche *et al.*, submitted). Thus, the v3-loop plays a major role in governing coreceptor choice, but does not impact CD4-dependence.

An important consequence of CD4-independence is enhanced susceptibility to antibody-mediated neutralization. HIV-1 IIIBx is approximately one-log more sensitive to neutralization by HIV-positive human sera and to rabbit sera raised against gp120 proteins than is the CD4-dependent parental virus strain (Hoffman *et al.*, 1999). The mechanism underlying this neutralization sensitive phenotype appears to be due to exposure of the highly conserved coreceptor binding site. Antibodies to this region neutralize IIIBx far more efficiently than they neutralize the CD4-dependent HIV-1 IIIB, while antibodies to the V3-loop neutralize both viruses equally well. Whether this phenotype can be taken advantage of to elicit broadly cross reactive neutralizing antibodies to this conserved region remains to be determined.

Finally, many primary SIV and HIV-2 virus strains exhibit CD4-independence (Edinger *et al.*, 1997; Endres *et al.*, 1996; Reeves *et al.*, 1997). In the case of SIV, many virus strains can use CCR5 to infect cells in the absence of CD4 (Edinger *et al.*, 1999; Edinger *et al.*, 1997). This suggests that the primordial receptor for the primate lentiviruses was CCR5 or a related molecule, and that the ability to use CD4 was acquired later. Since CD4-

independence appears to be associated with exposure of the conserved coreceptor binding domain, acquisition of the ability to use CD4 might make it possible to sequester this region until just before virus entry, protecting this site from neutralizing antibodies.

6. IMPLICATIONS FOR THERAPEUTIC INTERVENTION AND CONCLUDING THOUGHTS

Identification of the HIV coreceptors coupled with detailed structural information on CD4, gp120, and gp41 has suggested several new anti-viral approaches. The remarkable resistance of individuals who lack CCR5 to virus infection without obvious side effects due to loss of CCR5 function suggests that CCR5 is an attractive antiviral target. Other seven trans-membrane domain receptors are targets of highly effective and specific drugs, making it likely that CCR5 antagonists can be developed. Increased understanding of how gp120 and CCR5 interact should assist in the design of small molecule inhibitors. Likewise, CXCR4 antagonists would also be desirable given the prevalence of viruses that use this coreceptor. Indeed, several CXCR4 inhibitors have already been described (Donzella *et al.*, 1998; Doranz *et al.*, 1997; Murakami *et al.*, 1997; Schols *et al.*, 1997). Whether coreceptor blockade will force HIV-1 to evolve to use other coreceptors is not known, though as noted above receptors other than CCR5 or CXCR4 have not been shown to support infection of primary cells.

In addition to the coreceptors, Env itself can be targeted. The fusion inhibitory peptide T20 shows that an inhibitor of membrane fusion can be highly effective *in vitro* (Kilby *et al.*, 1998). The high resolution structure of gp41 and enhanced understanding of the mechanism by which T20 works may suggest regions of gp41 that can be targets for rationale drug design (Chan and Kim, 1998). The coreceptor binding site as well as the CD4 binding site on gp120 may also be targets for small molecule inhibitors. Interestingly, advances in understanding Env-mediated membrane fusion may also have implications for vaccine development. Recently, LaCasse *et al.* showed that immunization of mice with "fusion competent" Env elicits surprisingly potent and broadly cross reactive neutralizing antibodies (LaCasse *et al.*, 1999). In their study, cells expressing Env were mixed with cells expressing CD4 and CCR5, and fusion was allowed to occur prior to formaldehyde fixation. Immunization with these fixed cells, which presumably contain triggered Env proteins in which both the coreceptor binding site as well as gp41 determinants are exposed, resulted in this impressive humoral response. This suggests that generation of triggered or partially triggered Env proteins may elicit antibodies to highly conserved but poorly

immunogenic domains that are competent to neutralize diverse virus isolates.

With regards to the fusion reaction itself, the cast of characters is now complete and studies designed to determine how four proteins, gp120, gp41, CD4, and a coreceptor, interact in such a way as to lead to lipid mixing and membrane fusion are more feasible. Important questions that remain to be addressed include the architecture of the fusion pore and the role of inter- and intratrimer cooperativity. How many CD4 and coreceptor molecules are required to activate an Env trimer, and how many trimers are required to cause membrane fusion? How does the fusion peptide interact with the target membrane, and what is the role of the helical hairpin structure in membrane fusion? With the discovery of the coreceptors, it should be possible to design experiments to answer these questions.

ACKNOWLEDGMENTS. We thank Trevor Hoffman for providing Figure 1. C. M. was supported by NIH Training Grant NS0718F. R. W. D. was supported by NIH AI35383, AI 40880, the Burroughs Wellcome Fund, and by an Elizabeth Glaser Scientist Award.

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Chapter 13.

Sulfhydryl Involvement in Fusion Mechanisms

David Avram Sanders

1. PROTEIN THIOLS—AN INTRODUCTION

1.1. Cysteine—A Special Residue

At first one might wonder about the wisdom of focusing on the role of a single type of amino-acid residue in such a central biological process as protein-mediated membrane fusion. Indeed, an initial response might consist of the words of the daughters of Jerusalem, “What is thy beloved more than another beloved? [Song of Songs 5:91” There are excellent reasons, however, for acknowledging the special roles that cysteine residues play in protein-mediated membrane fusion. One important distinguishing feature is their sensitivity to the oxidation/reduction potential of their environment. It would not be inaccurate to state that the major difference between proteins that are within the cytoplasm and those that are topologically outside of the cell (this includes proteins and domains of proteins that are located in the lumens of eukaryotic secretory organelles or the periplasms of Gram-negative eubacteria) is that the cytoplasmic cysteines

DAVID AVRAM SANDERS Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907.

Subcellular Biochemistry, Volume 34: Fusion of Biological Membranes and Related Problems, edited by Hilderson and Fuller. Kluwer Academic / Plenum Publishers, New York, 2000.

possess thiols (except in the few cases when the thiol is posttranslationally modified), whereas the extracellular cysteines are found predominantly in cystine bridges.

Another important characteristic of cysteine is that its sulfhydryl group (a term that will be used interchangeably with thiol) is commonly the most reactive protein functional group. The sulfur atom is quite nucleophilic and therefore can be modified by a number of reagents (Lundblad, 1991). It is therefore relatively straightforward to probe the roles of cysteine residues in the function of a given protein or process. In addition, cysteine residues are highly conserved in protein sequences virtually exclusively because of the reactive sulfur. The interpretation of mutagenesis experiments in which conserved cysteine residues have been replaced is greatly simplified by the knowledge that it is unlikely that the cysteine is participating in some unique way in secondary-structure or hydrophobic-core formation. Finally, our interest is justified by existing data on the special roles of thiols and disulfides and their interchange in protein-mediated membrane fusion.

1.2. Oxidation and Reduction—Environment and Enzymatic Catalysis

From the start one must recognize that an evaluation of the roles of cysteine residues in membrane fusion requires a distinction between two separate problems, membrane fusion in a reducing environment and membrane fusion in an oxidizing environment. Proteins that are likely to promote membrane fusion during intracellular vesicular transport in eukaryotic cells reside in the reducing environment of the cytoplasm, whereas those participating in the promotion of membrane fusion between enveloped viruses and cells or between two or more cells (sperm-egg fusion, syncytium formation resulting from fusion of myoblasts into myotubes, etc.) are exposed to an oxidizing milieu. Therefore, the functions played by cysteine residues under the two conditions and the analytical techniques used to investigate them may be quite different.

A clear example of this principle is that the formation, rearrangement, and reduction of disulfide bonds are the most common events involving cysteine residues in extracellular proteins or protein domains, whereas they are uncommon in cytoplasmic proteins. Sequence analyses and residue-substitution experiments with membrane-bound or extracellular proteins must therefore focus on pairs of cysteine residues. The cystine bridges formed from these pairs are frequently critical to the integrity of the folded protein in the extracellular and secretory organelle lumenal environment. Incompletely folded or unstable proteins tend to be processed inefficiently or are retained intracellularly and degraded by the secretory system (Hammond

and Helenius, 1995). Determination of the specific role of a particular disulfide linkage may therefore not be amenable to mutational analysis.

In addition, the elimination of just one of a pair of cysteine residues that form a disulfide in the wild-type protein would leave a free thiol. Exposed free thiols in an extracellular protein domain generally result in retention of the protein in the endoplasmic reticulum (Fra *et al.*, 1993; Isidoro *et al.*, 1996; Lodish and Kong, 1993; Reddy *et al.*, 1996). This is an important aspect of the protein-folding quality control that the secretory system imposes on proteins that enter it. The molecular logic is the following: Virtually all cysteine residues in extracellular domains are present in disulfide bonds. Disulfide-bond formation does not, however, dictate the folded conformation of a protein. The cystine bridges present in the mature protein form between cysteine residues that are brought into juxtaposition by the same thermodynamic forces that promote the proper folding of cytoplasmic proteins. Therefore, the formation of disulfide bonds and the consequent removal of free thiols from the protein surface is an excellent indication that the protein has reached its final folded state and is thus prepared to proceed to its ultimate destination.

There are three points relating to the foregoing that are worth addressing and that are relevant to the discussion of the roles of thiols in protein-mediated membrane fusion. First, although disulfide-bond formation between two cysteine residues can occur spontaneously under oxidizing conditions, thiol-disulfide exchange enzymes catalyze the formation and reduction of cystine bridges in proteins that are topologically extracellular. These enzymes are referred to as protein disulfide isomerases in eukaryotes and Dsb proteins in prokaryotes (Bardwell, 1994; Freedman *et al.*, 1994). Whereas catalysis of disulfide-bond formation occurs during the process of protein exit from a cell, disulfide-bond reduction appears to be required during the entrance of certain proteins into a cell.

A consideration of some examples of disulfide-bond reduction during entry possesses relevance for our treatment of the role of disulfides in membrane fusion. Evidence has been presented for the reduction of protein disulfides during processing of antigens, such as insulin, that are taken up into cells and presented by MHC class II molecules (Jensen, 1991). Studies of the entry of toxins into cells provide additional examples. Cholera toxin consists of two subunits (A and B). The A subunit is cleaved into two fragments (A1 and A2) linked by disulfide bonds. Cholera toxin is trafficked by retrograde transport through the secretory system to the endoplasmic reticulum where the reduction of the disulfide bonds takes place. As a result the A1 fragment, which possesses the ADP-ribosyltransferase activity of the toxin, is released (Majoul *et al.*, 1996). Ricin and diphtheria toxin consist of two subunits that are linked by interchain disulfide bonds. Reduction of the

disulfide bond appears to be a rate-limiting step in membrane penetration by both toxins (Lewis and Youle, 1986; Papini *et al.*, 1993)). The cytotoxic effects of exposing cells to diphtheria-toxin, but not ricin, can be prevented through incubation of the cells with reagents, such as 5,5'-dithiobis-(2-nitrobenzoic) acid and p-chloromercuriphenylsulfonic acid, that inhibit cell-surface protein-disulfide-isomerase activity (Ryser *et al.*, 1991). It is thus evident that such enzymatic activities need to be taken into consideration when evaluating the oxidation state of cysteines in membrane-fusion-promoting proteins.

The second point is that disulfide-bond formation between cysteine residues that are not linked in the final folded structure can occur during the process of protein folding. Normally, this would result both in the temporary stabilization of a partially folded or misfolded state of the protein and in the presence on the protein surface of free thiols pertaining to the two cysteine residues whose partners in the final folded state are for the moment linked in an unsanctioned union. A eukaryotic protein bearing aberrant disulfide bond(s) would tend to be retained in the endoplasmic reticulum where protein-disulfide-isomerase could reduce the cystine bridge. Refolding of the proteins could then take place, and the appropriate disulfide linkages could consequently be made. The probable reasons that there is not an infinite cycle of oxidation and reduction for the correct disulfide bonds are that the folded proteins rapidly exit from the compartment where the protein disulfide isomerase is localized and that a disulfide bond is more hydrophobic than are a pair of thiols, so once it has formed, and the tertiary and quaternary structure of the protein are stabilized, the cystine bridge is unlikely to remain exposed to the protein disulfide isomerase.

Finally, unpaired cysteines in extracellular protein domains are rare but they do exist (Reddy *et al.*, 1996). In almost all cases they are not absolutely conserved among homologous proteins and virtually all of those that have been investigated are not exposed on the surface. One of the rare exceptions to the rule about the absence of conservation of an odd number of cysteine residues in the extracellular domains of a homologous family of proteins will be considered below. It is the very rarity of such an anomaly that contributed to the recognition of its importance in a class of proteins that promotes membrane fusion.

1.3. Thiol and Disulfide Modification Reagents

Part of our appreciation of the roles of thiols in membrane fusion arises from studies involving the chemical modification of protein cysteine residues. A thorough comprehension of the implications of such studies can

only be achieved through an in-depth understanding of the reactions involved. It is the goal of the following brief discussion to familiarize the reader with the chemistry of the commonly used reagents with an emphasis on the nature of the reactive cysteine-containing species, the product of the reaction, and the reversibility of the modification. Technical aspects of the use of the reagents will not be considered.

Cysteine residues differ from most functional groups on a protein in that they can be readily oxidized, alkylated, acylated, cyanylated, or arylated (Lundblad, 1991). It should be noted that the acid dissociation constant (pKa) for an average cysteine residue in a polypeptide is approximately 8.5 and that the thiolate anion is more reactive with modifying reagents than is the free thiol. Cysteine residues in active sites often have lowered pKas and, therefore, increased concentration as the thiolate anion. Consequently, they are frequently more rapidly modified than other cysteine residues.

Among the reagents that have achieved the most widespread use for modification of protein thiols are the α -haloacetic acids, such as iodoacetic acid and chloroacetic acid, and α -haloacetamides, such as iodoacetamide and chloroacetamide (Lundblad, 1991). The product of the reaction of a cysteine residue with iodoacetic acid or iodoacetamide is a stable carboxymethylated or carboxamidomethylated cysteine. It is important to keep in mind that histidine residues can also be carboxymethylated by iodoacetic acid.

Monobromobimane and its derivatives are very useful reagents for fluorescent labeling of proteins containing thiols (Kosower and Kosower, 199%). Nucleophilic attack by the thiolate on the nonfluorescent bromobimane displaces the bromide ion and results in a fluorescently labeled cysteine residue.

Other popular reagents are *N*-ethylmaleimide and its derivatives. Conditions can normally be found where they react specifically with cysteine residues (Lundblad, 1991). Upon acid hydrolysis of a thiol-containing protein modified with *N*-ethylmaleimide, *S*-succinyl cysteine is obtained. Important characteristics distinguishing modifications by the α -haloacetic acids, α -haloacetamides, *N*-ethylmaleimide and maleimide derivatives and those resulting from reactions with some other thiol reagents are that the former modifications are essentially irreversible and that the reagents cannot catalyze disulfide-bond formation.

In contrast, the modification of a cysteine residue with 5,5'-dithiobis-(2-nitrobenzoic) acid, which leads to the formation of a mixed disulfide in which the cysteine is linked to a 2-mercapto-5-nitrobenzoic acid, can be readily reversed by addition of a reducing reagent such as dithiothreitol and can lead to formation of a protein cystine (Lundblad, 1991). *N*-succinimidyl

3-(2-pyridyldithio)propionate (SPDP) is a heterobifunctional reagent, whose hydroxysuccinimide ester reacts with amino groups to form an amide bond and whose 2-pyridyldisulfide group reacts with thiols to form a disulfide (Jou *et al.*, 1983). Through use of SPDP one can link a molecule that contains an amino group to a protein thiol.

Other useful reagents include 2-nitro-5-thiocyanobenzoic acid, which can cyanylate cysteine residues, and organic mercurials, such as mersalyl acid (*o*-[(3-Hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetic acid), *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid, which can bond to the cysteine sulfur atom through the mercury atom (Lundblad, 1991). Of particular interest are the facts that peptides can be cleaved at *S*-cyano residues under alkaline conditions and that the organomercurials can be displaced by thiol-containing reagents. It is also worth noting that *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid are converted to the active reagents *p*-hydroxymercuribenzoic acid and *p*-hydroxymercuriphenylsulfonic acid when the solids are dissolved in water.

Aspergillus japonicus produces a potent inhibitor specific for cysteine proteases, E-64, which was determined to be an *L-trans*-epoxysuccinylleucylagmatine derivative (Katunuma and Kominami, 1995). It and other subsequently synthesized *L-trans*-epoxysuccinylpeptides have been shown to form covalent thioether bonds with the active-site cysteines of the proteases. Compounds specific for particular cysteine proteases have been synthesized.

A number of reagents have been used to examine the effects of oxidizing cysteines to form cystine bridges. Sodium periodate has been reported to be a mild agent that oxidizes only vicinal cysteines to disulfides (Rippa *et al.*, 1981). Diamide (diazenedicarboxylic acid bis(*N,N*-dimethylamide)) reacts with thiols in two stages (Kosower and Kosower, 1995a). In the first, the thiolate adds to the diazene double bond to form a sulfenylhydrazine, which, in the second step reacts with a second thiolate to yield a disulfide and a hydrazine. Molecular iodine and Cu(II)(1,10-phenanthroline)₃ are both used to oxidize cysteine residues to disulfides but have different properties. Iodine is a reactant that promotes rapid oxidation and is consumed during the process, whereas Cu(II)(1,10-phenanthroline)₃ is a catalyst that promotes a slower oxidation (Hughson *et al.*, 1997). Sodium tetrathionate is also commonly used to promote the formation of cystine bridges from protein thiols.

Protein disulfides can be reduced by a number of reagents, the most common of which are 2-mercaptoethanol, dithiothreitol, and dithioerythritol. The reagents are not completely interchangeable. Dithiols, such as dithiothreitol, and monothiols, such as 2-mercaptoethanol, are of

comparable effectiveness for reduction of an intermolecular disulfide. However, a dithiol is kinetically superior as a reductant for intramolecular cystine bridges when compared to a monothiol (Gilbert, 1990). This fact may underlie the very different *in vivo* effects of dithiothreitol treatments versus 2-mercaptoethanol treatments upon the secretion from mammalian cells of proteins containing cysteine residues and/or cystine bridges (Valetti and Sitia, 1994).

2. PROTEIN THIOLS IN CELLULAR MEMBRANE FUSION

2.1. Identified Thiol-Reagent-Modified Proteins

2.1.1. *N*-ethylmaleimide-Sensitive Factor-NSF

The most prominent protein bearing a thiol whose modification interferes with cellular membrane-fusion processes is the *N*-ethylmaleimide-sensitive factor (NSF). A complete treatment of the roles of NSF in secretory vesicular transport and its interactions with soluble NSF attachment proteins (SNAPs) and SNAP receptors (SNAREs) is beyond the scope of this review. Numerous recent reviews of the function of these proteins are available (Clague, 1998; Hay and Scheller, 1997; Nichols and Pelham, 1998; Robinson and Martin, 1998; Woodman, 1997). However, consideration of a number of points concerning the effects of *N*-ethylmaleimide treatment upon its function will be instructive. It is also important to be familiar with some of the characteristics of NSF, because in publications that will be discussed below the properties of other membrane-fusion factors are explicitly contrasted with those of NSF.

Biosynthetic transport between Golgi cisternae in cell-free systems is inhibited by treatment with 1 mM *N*-ethylmaleimide and restored by addition of untreated NSF, which appears to be the inactivation target in the membranes. NSF is a hexameric ATPase that plays a central role in many vesicular transport pathways including endoplasmic reticulum-Golgi apparatus transport, transport between Golgi complex cisternae, endosomal vesicle fusion, and transport from the Golgi to specialized membranes in polarized cells (Woodman, 1997). The membrane-fusion-promotion and ATPase activities of purified NSF are inhibited by treatment with 2 mM *N*-ethylmaleimide (Block *et al.*, 1988; Tagaya *et al.*, 1993). NSF forms an ATP-dependent complex with soluble NSF attachment proteins (SNAPs) that in turn interact with SNAP receptors (SNAREs) and, it is believed, thereby induce conformational changes that affect SNARE function (Woodman, 1997). It has been recently suggested that NSF may also act as a molecular

chaperone for other proteins besides those involved in membrane fusion (Haas, 1998). There are two ATPase domains in NSF (Tagaya *et al.*, 1993), referred to as D1 and D2, which differ in affinity for ATP (Matveeva *et al.*, 1997). The low-affinity sites, which are on the D1 domains, appear to be critical for NSF to associate with the SNAP-SNARE complex (Matveeva *et al.*, 1997; Whiteheart *et al.*, 1994).

It has been suggested that a cysteine residue in the β -phosphate binding loop of D1 contains the thiol that reacts with *N*-ethylmaleimide (Tagaya *et al.*, 1993). Consistent with this hypothesis is the fact that the yeast homologue of NSF, Sec18p, has a threonine residue at the corresponding position and is resistant to inactivation by *N*-ethylmaleimide (Steel *et al.*, 1999). However, it would seem prudent if the site of modification were determined directly; *N*-ethylmaleimide treatments that abolish ATPase activity do not appear to inhibit ATP binding, as might have been expected if the modification were in the β -phosphate binding loop (Matveeva *et al.*, 1997).

2.1.2. Calpains

It would appear that the most prominent example of the effects of thiol-reagent treatments upon membrane fusion is a protein whose presumed modified cysteine is not, in fact, essential for function; it is the modification that is inhibitory. Potential targets of thiol-modifying reagents that do possess active-site cysteine residues are the calpains (intracellular, Ca^{2+} -dependent thiol proteases). The possibility of the participation of calpains in various membrane-fusion processes has been investigated. The membrane mobility agent 2-(2-methoxyethoxy)ethyl-*cis*-8-(2-octylcyclopropyl)octanoate (A_2C) promotes fusion of rat erythrocytes in the presence of Ca^{2+} . Cell fusion and the Ca^{2+} -dependent membrane-protein degradation that appears to precede it are prevented by pretreatment of the erythrocytes with *N*-ethylmaleimide or monobromobimane (Glaser and Kosower, 1986). In contrast, there was no effect when the cells were preincubated with pepstatin, phenylmethylsulphonyl fluoride, or 1,10-phenanthroline, which are inhibitors, respectively, of aspartyl proteases, serine proteases, and metalloproteases. Erythrocyte ghosts undergo A_2C and Ca^{2+} -dependent cell fusion and membrane-protein degradation solely when they contain hemolysate. Cell fusion and membrane-protein degradation are not detected with erythrocyte ghosts reconstituted with hemolysate treated with *N*-ethylmaleimide or monobromobimane (Glaser and Kosower, 1986). A partially purified proteolytic activity, that possessed the biochemical and chromatographic characteristics of calpain I, when loaded with Ca^{2+} into the

erythrocyte ghosts could restore cell fusion in the presence of A_2C (Glaser and Kosower, 1986).

The same laboratory has probed the role of calpain in myoblast fusion into multinucleated myotubes. It was demonstrated that whereas the level of calpain polypeptide does not undergo major changes during rat L8 myoblast differentiation and fusion, the level of calpastatin, the endogenous inhibitor of calpain, decreased dramatically during the stages before and during myoblast fusion (Barnoy *et al.*, 1996). Calpain activity was stimulated during this period of time. Again, when calpain activity was inhibited either directly, by inhibitors such as calpeptin or E-64d, or indirectly, through incubation of the myoblasts with TGF- β (which appears to prevent the diminution of calpastatin levels that accompanies myoblast differentiation), protein degradation within the myoblasts and myoblast fusion are inhibited (Barnoy *et al.*, 1997; Barnoy *et al.*, 1998).

2.2. Experimental Systems with Thiol-Reagent- or Disulfide-Reagent-Modified Proteins of Unknown Identity

2.2.1. Frog Neuromuscular Junction

There are numerous systems where protein thiols have been implicated in cellular membrane-fusion events, but the targets of modification have not been identified. In the amphibian neuromuscular junction the thiol-oxidizing agent diamide stimulates the frequency and amplitude size of miniature endplate potentials (transmitter release) in a temperature-dependent fashion (Publicover and Duncan, 1981). The stimulation is reversed by incubation of the frog cutaneous pectoris nerve-muscle preparation with dithioerythritol and does not appear to be acting through effects on mitochondria. The authors conclude that oxidation of protein thiols promotes vesicle-plasma membrane fusion (Publicover and Duncan, 1981).

2.2.2. Mammalian Sperm-Egg Fusion

Mammalian fertilization culminates with the fusion of a spermatozoon that has penetrated the zona pellucida with the plasma membrane of an egg. The effects of the reactive oxygen species, superoxide and hydrogen peroxide, on mouse sperm motility and sperm-egg fusion have been investigated. The reactive oxygen species were generated by addition of xanthine oxidase at various concentrations to hypoxanthine; the higher the concentration of xanthine oxidase the higher the level of the reactive oxygen species. At high levels of these species sperm motility and sperm-egg fusion

were decreased, and lipid peroxidation was increased (Mammoto *et al.*, 1996). Sperm motility was not restored by incubation with 1mM dithiothreitol. At lower levels of reactive oxygen species, sperm-egg fusion was still greatly inhibited, although sperm motility and lipid peroxidation were not perturbed. Preincubation of the sperm with both superoxide dismutase and catalase prevented the inhibition of sperm-egg fusion by the oxygen species, whereas incubation with only one or the other of the reagents had only a partial protective effect. Most significantly, 250 mM dithiothreitol completely reversed the effects of the xanthine oxidase treatment on sperm-egg fusion (Mammoto *et al.*, 1996).

The effects of treatments with *N*-ethylmaleimide, sodium tetrathionate and 5,5'-dithiobis-(2-nitrobenzoic) acid on sperm-egg fusion have also been investigated. These reagents inhibited fertilization without affecting sperm motility (Mammoto *et al.*, 1997). It was demonstrated that the effect of *N*-ethylmaleimide treatment was not upon penetration of the zona pellucida by the sperm, nor was it upon either sperm-egg binding or the sperm acrosome reaction. Sperm nuclear incorporation into eggs was reduced by the thiol-modifying reagents, so it was concluded that sperm-egg fusion was specifically inhibited (Mammoto *et al.*, 1997). Four additional results were described:

1. Treatment of the eggs with the thiol reagents did not inhibit sperm-egg fusion, so the targets of modification appeared to be in the sperm.
2. The effects of treatment with the oxidizing sodium tetrathionate were reversed by incubation with DTT, suggesting that oxidation of protein thiols was involved in the inhibition of sperm-egg fusion.
3. The membrane-impermeant thiol reagent, eosin-5-maleimide, failed to inhibit sperm-egg fusion, suggesting that the site of action of the other reagents was either intracellular or intramembrane.
4. Putative sperm protein targets of the thiol modifications were identified (Mammoto *et al.*, 1997).

2.2.3. Insulin and Renin Secretion

Much earlier research was directed towards investigating the effects of thiol-modifying reagents on the release of insulin resulting from the fusion of secretion granules with the pancreatic islet cell plasma membrane. Various models were proposed to explain the stimulation of insulin release by reagents such as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide. The potential importance of membrane-protein thiol-disulfide exchange and nicotinamide adenine dinucleotides was emphasized. Because of the lack

of currency of the literature, I refer the interested reader to an article (from one of the laboratories in which the issues involved have been examined most extensively) that reviewed the state of the field (Cooperstein and Watkins, 1990).

More recent investigations have been conducted on the inhibition by thiol reagents of renin secretion from rabbit renal cortical slices. The poorly membrane-permeant reagents *p*-chloromercuriphenyl sulfonate (500 μ M) and stilbene maleimide (1 mM) stimulated renin secretion (95-fold and 15-fold respectively), whereas the membrane-permeant *N*-ethylmaleimide did not (Doh *et al.*, 1991). Incubation of the renal cortical slices with 5mM dithiothreitol had no effect upon renin secretion, but it prevented the stimulation of secretion by *p*-chloromercuriphenyl sulfonate. Incubation with 10mM dithiothreitol inhibited renin secretion by 86% (Doh *et al.*, 1991). Inhibition by stilbene maleimide was not reversible. Peculiarly, it was found that the thiols that reacted with *p*-chloromercuriphenyl sulfonate were not accessible to *N*-ethylmaleimide (Doh *et al.*, 1991), although they would appear to have reacted with the stilbene maleimide. Perhaps the membrane-permeant *N*-ethylmaleimide was consumed through reaction with cytoplasmic glutathione (Doh *et al.*, 1991). Stimulation of renin secretion (50-fold) by 1 mM mersalyl was also detected; stimulation was reversed by incubation with 5 mM dithiothreitol (Park *et al.*, 1991). It was concluded that thiol-disulfide exchange in proteins on the extracellular side of the membrane played an important role in renin secretion (Doh *et al.*, 1991).

2.2.4. Sea Urchin Pronuclear Fusion during Fertilization

Studies of sea urchin eggs yielded evidence for effects of thiol-disulfide-modifying reagents on a number of different membrane-fusion steps. When sea urchin eggs are treated with 1 mM dithiothreitol three minutes after fertilization, pronuclear fusion does not take place, although pronuclear migration and chromosome condensation and decondensation are not affected (Schatten, 1994). Mitosis continues in a fashion, but proper karyomere membrane fusion and division-furrow construction are not achieved. It was concluded that the maintenance of some protein disulfide-bond(s) is necessary for the three membrane-fusion events to transpire normally. The roles of thiols and disulfides in fertilization and cell division are also reviewed (Schatten, 1994).

2.2.5. Sea Urchin Egg Cortical Granule Exocytosis

Sea urchin egg cortical granule exocytosis is a regulated response triggered by the rise in Ca^{2+} concentrations that accompanies fertilization. The

cortical vesicles that fuse with the plasma membrane are tightly associated with its cytoplasmic face in the mature egg before fertilization. Fragments of egg cortex that contain the cortical vesicles and the plasma membrane can be utilized as a cell-free system for studying Ca^{2+} -induced exocytosis (Vogel *et al.*, 1991). The cortical vesicles and plasma membranes can also be isolated from one another and then recombined to reconstitute Ca^{2+} -triggered exocytosis (Vogel *et al.*, 1991). *N*-ethylmaleimide treatments inhibit the fusion step in both the egg-cortex-fragment and reconstituted systems (Jackson and Modern, 1990). The *N*-ethylmaleimide-sensitive proteins appeared to be firmly associated with the membranes (Jackson and Modern, 1990).

Interestingly, reconstitution of exocytosis could be achieved by recombination of *N*-ethylmaleimide-treated plasma membrane with untreated cortical vesicles or of *N*-ethylmaleimide-treated cortical vesicles with untreated plasma membrane, whereas the combination of *N*-ethylmaleimide-treated plasma membrane and *N*-ethylmaleimide-treated cortical vesicles was inactive (Jackson and Modern, 1990). These data suggested that the *N*-ethylmaleimide-sensitive protein(s) resided in both the plasma membrane and cortical vesicles and could be supplied by either one (Jackson and Modern, 1990).

It has been demonstrated that the cortical granules will themselves fuse in the presence of Ca^{2+} in a reaction that is inhibited by *N*-ethylmaleimide (Vogel *et al.*, 1992; Vogel and Zimmerberg, 1992). Untreated vesicles will fuse with *N*-ethylmaleimide-treated vesicles, thereby demonstrating that the *N*-ethylmaleimide-sensitive proteins that promote fusion need be on only one of the fusing membranes (Vogel *et al.*, 1992). There appears to be no requirement for cytoplasmic proteins in the fusion reaction. In addition, the cortical granules fuse with liposomes in an *N*-ethylmaleimide-inhibitable fashion (Vogel *et al.*, 1992).

Additionally, it has been demonstrated that treatment of the membranes with 3-(2-pyridyldithio)propionate coupled to dextran (dextran-PDP) inhibited granule exocytosis in a reaction that was reversible by subsequent addition of dithiothreitol (Whalley and Sokoloff, 1994). The dextran-PDP appeared to react with the same thiols as the *N*-ethylmaleimide, because it prevented the irreversible inhibition of fusion by *N*-ethylmaleimide. These data suggested that the functionally significant thiols are readily exposed on the membranes.

2.2.6. Microsome Fusion

Homotypic fusion of microsomes derived from disrupted rat liver endoplasmic reticular membranes is dependent on GTP and inhibited by

incubation of the membranes with *N*-ethylmaleimide. By a number of criteria the *N*-ethylmaleimide-sensitive component was demonstrated to be distinct from NSF (Sokoloff *et al.*, 1995). Subjecting the untreated membranes to a procedure known to extract NSF from membranes did not affect microsome fusion, whereas addition of cytosol that had not been treated with *N*-ethylmaleimide did not restore fusion between *N*-ethylmaleimide-treated microsomes. NSF was not detected immunologically in microsomes that retained fusion capacity.

Treatment of the membranes with 3-(2-pyridyldithio)propionate coupled to bovine serum albumin (BSA-PDP) inhibited microsome fusion in a reaction that was reversible by subsequent addition of dithiothreitol (Sokoloff *et al.*, 1995). The BSA-PDP appeared to react with the same thiols as the *N*-ethylmaleimide, because it prevented the irreversible inhibition of fusion by *N*-ethylmaleimide. These data suggested that the functionally significant thiols are highly accessible on the membranes. Inhibition of fusion could also be achieved through treatments with sodium periodate in a reaction that was also reversible by subsequent addition of dithiothreitol. Incubation of the membranes with 1 mM Mg²⁺-GTP did not protect the fusion activity of the membranes from inhibition, thereby indicating that the reactive thiol was unlikely to be in a GTP-binding site (Sokoloff *et al.*, 1995).

2.2.7. Endocytosis

N-ethylmaleimide-sensitive factors that differ from NSF have been reported to participate in vesicular transport and membrane fusion during endocytosis. In a cell-free system that examines the transport of mannose 6-phosphate receptors from late endosomes to the trans-Golgi network, it has been demonstrated that transport was 80% inhibited when mixtures of cell extracts and Golgi membranes were treated with 0.2 mM *N*-ethylmaleimide (Goda and Pfeffer, 1991). Transport was largely restored when untreated, but not *N*-ethylmaleimide-treated, cytosol was added to the mixture. Glycerol-gradient sedimentation analysis of the active component of the cytosol, dubbed ETF-1, indicated that it had a molecular mass of 50–100 kDa, whereas NSF is a hexamer of 76-kDa subunits (Fleming *et al.*, 1998). Furthermore, the ETF-1 activity was still present in cytosol depleted of NSF by passage over columns containing bound anti-NSF antibodies. In addition, levels of *N*-ethylmaleimide that inhibited endosome → trans-Golgi network transport did not inhibit NSF-dependent intra-Golgi transport indicating that NSF is not the target of *N*-ethylmaleimide in the endosome → trans-Golgi network transport system (Goda and Pfeffer, 1991). ETF-1 activity was found to be required for an early stage in transport but has not been further characterized.

Fusion between endosomal vesicles was inhibited 75% when 1 mM *N*-ethylmaleimide was incubated with a mixture of membrane and cytosol fractions (Rodriguez *et al.*, 1994). When the fractions were incubated separately with 1 mM *N*-ethylmaleimide and then combined, fusion was not diminished substantially. When the fractions were incubated with 3 mM *N*-ethylmaleimide either together or separately and then combined, vesicular fusion was almost completely eliminated. Addition of untreated cytosol, but not untreated membranes, could restore fusion in mixtures treated with either 1 mM or 3 mM *N*-ethylmaleimide. Immunological analysis of the fractions indicated that NSF was found mainly in the membrane fraction, and was therefore unlikely to be the active cytosolic component (Rodriguez *et al.*, 1994). Recombinant NSF that is active in intra-Golgi transport assays restored fusion only slightly. On the other hand, when treatments known to deplete NSF activity from the cytosol (incubation for 30 minutes at 37°C after desalting in the absence of ATP) were conducted, the fusion restoration activity of the cytosol was not substantially reduced. It was concluded that there is a cytosolic *N*-ethylmaleimide-sensitive factor involved in endosomal vesicle fusion that differs from NSF, although NSF itself is also required. It is also proposed that the thiol on the second factor is exposed only upon interaction of the protein with the membrane (Rodriguez *et al.*, 1994).

3. PROTEIN THIOLS IN VIRAL-GLYCOPROTEIN-MEDIATED MEMBRANE FUSION AND VIRUS ENTRY

3.1. Human Immunodeficiency Virus

Much of the best evidence for the role of thiols and, specifically, thiol-disulfide exchange in membrane fusion comes from studies of the entry of enveloped viruses and viral glycoproteins. However, the solidity of the support for the participation of thiol-disulfide exchange varies considerably. The largest body of data buttressing the role of cell surface thiol-disulfide exchange proteins in promoting the entry of an enveloped virus concerns the abrogation of human immunodeficiency virus type 1 (HIV-1) replication by inhibitors of cell-surface protein disulfide isomerase activity. Although protein disulfide isomerase is normally considered an endoplasmic reticulum-localized protein (Freedman, 1989), cell-surface protein disulfide isomerase has been detected by, for example, flow cytometric analysis utilizing anti-protein disulfide isomerase monoclonal antibodies (Ryser *et al.*, 1994). These antibodies also substantially inhibited HIV-1 infection of human T-cells, H9 (HUT-78). In addition, treatment of the cells

prior to infection with 2.5 mM 5,5'-dithiobis-(2-nitrobenzoic) acid, 1 mM monobromotrimethylammoniumbimane, or 3 mM bacitracin (an inhibitor of protein disulfide isomerase (Mandel *et al.*, 1993)), dramatically inhibited HIV-1 infection (Ryser *et al.*, 1994). Effects of these three reagents on cell-surface expression of the HIV-1 coreceptor CD4 and on cell viability were negligible. Treatment of the cells with 2.5 mM 5,5'-dithiobis-(2-nitrobenzoic) acid following initial HIV-1 infection but before transfer to regular growth medium did not reduce viral replication. Pretreatment of the virus with the reagents prior to binding did not inactivate the virus (the treated virus was diluted so that the concentration of the inhibitors was much lower than their effective concentrations when the virus was incubated with the cells). In addition, exposure of HIV-1 to dithiothreitol decreased HIV infectivity in a dose-dependent manner. It was therefore concluded that a cell-surface protein disulfide isomerase was involved in promoting HIV-1 entry, presumably by reducing a critical disulfide bond upon receptor binding (Ryser *et al.*, 1994).

3.2. Coronaviruses

Murine hepatitis virus, a coronavirus, possesses mature glycoprotein spikes that consist of two subunits, S1 and S2, which are responsible for both the binding of virions to cell membranes and virion-cell membrane fusion. S2 is a transmembrane protein, whereas S1 is associated with the viral envelope through binding to S2. It was demonstrated that incubation of the JHM strain of MHV with 1 mM 5,5'-dithiobis-(2-nitrobenzoic) acid at 37°C for one hour reduced infectivity by 99%, whereas incubation at 21°C or incubation of the A59 strain with 1 mM 5,5'-dithiobis-(2-nitrobenzoic) acid did not affect infectivity (Gallagher, 1996). The effect on the JHM strain resulted from reduced viral-cell fusion and not from diminished binding of the virus to the cell. It was noted that the S2 of the JHM strain possesses 13 cysteine residues and the S2 of strain A59 contains 12, presumably paired in six disulfides. The 13th S2 cysteine in JHM is not involved in covalent intermolecular associations, so it is likely to exist as a thiol. This residue (cysteine-1163) was altered to tyrosine, the residue in the equivalent position in A59, and the recombinant S protein was assayed in a cell-cell fusion assay. The mutation greatly reduced fusion potency, but it also reduced sensitivity to 1 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (Gallagher, 1996). Introducing the cysteine residue into the A59 strain S2 increased sensitivity to the reagent. It was concluded that cysteine-1163 is exposed at higher temperatures, and that its modification can lead to an inhibition of fusion (Gallagher, 1996). The fact that the cysteine residue is not required for fusion activity reduces the likelihood that it is involved in

thiol-disulfide-exchange-induced conformational changes that promote membrane fusion. It had previously been suggested that such exchange might be important for coronavirus glycoprotein-induced membrane fusion because of the enhancement of S1 release and virus aggregation by dithiothreitol treatment of A59 strain virions and because of the possibility that these conformational changes were related to those that accompany fusion (Sturman *et al.*, 1990). In conclusion, it is unlikely that the 5,5'-dithiobis-(2-nitrobenzoic) acid-sensitivity of JHM strain virions is related to the effects of dithiothreitol treatment of the A59 strain virions. Further studies are required to determine whether thiol-disulfide exchange plays a role in coronavirus entry.

3.3. Alphaviruses

A series of investigations into the roles of disulfide bonds and thiol-disulfide exchange in the structure of alphavirus glycoproteins and their promotion of membrane fusion have been conducted. The glycoproteins of the alphaviruses are synthesized as a polyprotein (E3-E2-6K-E1), which is processed into the individual subunits (Strauss and Strauss, 1994). The amino-terminal section of the polyprotein functions as a signal sequence to direct the translocation of the subsequent polypeptide region into the endoplasmic reticulum. A hydrophobic sequence approximately 400 residues after the signal sequence acts as a stop-transfer signal and as the membrane anchor for E2. The following segment of approximately 30 residues transiently functions as a signal sequence for the carboxy-terminal half of the molecule. Proteolytic cleavage following this signal sequence results in the release of E3-E2 (referred to as proE2 or PE2), which is anchored in the membrane. Other processing events lead to the formation of the trans-membrane E1 protein.

PE2-E1 heterodimers form in the endoplasmic reticulum and are transported to the Golgi apparatus where PE2 is cleaved into E3 and E2 at a sequence recognized by the furin class of protein convertases (Strauss and Strauss, 1994). Cleavage of PE2 to E3 and E2 appears to be required for normal infectivity of alphaviruses in mammalian cells (Strauss and Strauss, 1994). The mature E2-E1 glycoprotein complex from the Sindbis virus envelope is stable in detergent but is disrupted by treatment with dithiothreitol or 2-mercaptoethanol (Anthony and Brown, 1991), although there is no evidence for stable intermolecular E1-E2 disulfide bonds. Incubation of Sindbis virus with 5 mM dithiothreitol induces the exposure of epitopes that are also recognized by certain monoclonal antibodies upon attachment of the virus to cells (Meyer *et al.*, 1992). Prolonged exposure of the virus to 5 mM dithiothreitol (6 hours) results in a dramatic decrease in

infectivity, an increase in the susceptibility of the glycoproteins to digestion with trypsin, and morphological changes (Anthony *et al.*, 1992). It was inferred that disulfide bonds are responsible for maintaining the structural integrity of the Sindbis virus envelope protein lattice (Anthony *et al.*, 1992).

Subsequent experiments on the role of thiol-disulfide exchange in Sindbis virus entry have figured into a controversy on the cellular site of entry of alphaviruses. Most of the data are consistent with the concept that Semliki Forest and Sindbis viruses enter cells through a clathrin-dependent pathway that involves acidified endosomes (DeTulleo and Kirchhausen, 1998; Glomb-Reinmund and Kielian, 1998; Marsh and Helenius, 1989; Strauss and Strauss, 1994). It has been suggested, however, that the reduction in infection by Sindbis virus resulting from incubation of cells with lysosomotropic weak bases is mediated through an inhibition of viral replication rather than through an abrogation of entry resulting from the inhibition of the acidification of endosomes (Cassell *et al.*, 1984).

There is an alternative hypothesis to the proposal that alphavirus entry occurs through the endocytic pathway. It postulates that thiol-disulfide exchange reactions at the cell surface lead to Sindbis virus-mediated fusion (Abell and Brown, 1993). The chain of reasoning begins with experiments that appear to support a role for acidic pH, that is, the endocytic pathway, for Sindbis virus-promoted membrane fusion. Exposure of Sindbis virus to pH 5.3 is necessary for its mediation of cell-cell fusion that occurs when the treated virus is present at high concentrations. However, fusion itself occurs efficiently only when the pH is raised towards neutrality. This was interpreted to mean that fusion could normally only take place at a pH where protein thiolates would be present. Based upon this hypothesis, it was demonstrated that if the cells were incubated with submicromolar concentrations of 2-mercaptoethanol, then fusion could occur efficiently at pH 5.3 (Abell and Brown, 1993). In addition, if cells to which virus had been adsorbed were incubated with 1 mM 5,5'-dithiobis-(2-nitrobenzoic) and thereafter warmed to allow virus penetration, then virus infectivity is inhibited 50–40%. By contrast, treatment of the virus and cells before or after the process of penetration had little effect. It was hypothesized that certain thiols and/or disulfides become accessible because of the conformational changes in the glycoproteins that occur during penetration (Abell and Brown, 1993; Meyer *et al.*, 1992). Blockage by 5,5'-dithiobis-(2-nitrobenzoic) acid of the thiols or those on a cellular protein that binds to the glycoproteins is proposed to be the mechanism by which membrane fusion is inhibited (Abell and Brown, 1993).

Additional studies of the nature of the disulfide bonds within the Sindbis virus glycoproteins possess potential relevance for understanding

the role of thiol-disulfide exchange in virus entry. Processing of the Sindbis E1 protein was investigated utilizing pulse-chase experiments and analysis of the migration of E1 in SDS-polyacrylamide gel electrophoresis under nonreducing and reducing conditions (Mulvey and Brown, 1994). Cells and virus were lysed in the presence of 20 mM *N*-ethylmaleimide in order to inhibit artifactual disulfide bond formation or rearrangement. Mobility of endoglycosidase H-treated E1 during SDS-polyacrylamide gel electrophoresis was also determined to examine whether a particular E1 species had left the endoplasmic reticulum and thereby acquired endoglycosidase H-resistant oligosaccharides. It was established that E1 in the endoplasmic reticulum exists in a number of species that differ in the number and/or arrangement of their disulfides. One oxidized form of E1, referred to as E1 ϵ , appeared to be competent for export from the endoplasmic reticulum. By a number of criteria, including analysis of the effects of brefeldin A treatments, which induces the return of Golgi components to the endoplasmic reticulum, E1 ϵ was demonstrated to be a stable entity during progress through the Golgi apparatus (Mulvey and Brown, 1994). Additional experiments indicated that E1 ϵ was resistant to 5-minute exposure to 5mM dithiothreitol *in vivo*, indicating that it had acquired a mature conformation (Carleton and Brown, 1996). However, late in the secretory pathway, E1 ϵ was found to be unstable with regard to its disulfide bonds. This instability was also found during analysis of E1 from Sindbis virions (Mulvey and Brown, 1994).

As noted above, a precursor to E2, PE2, associates with E1 in the endoplasmic reticulum. No disulfide rearrangements of PE2 or E2 were detected during an analysis of their processing (Mulvey and Brown, 1994). Association of PE2 with E1 in the endoplasmic reticulum can be disrupted by *in vivo* dithiothreitol treatments, but while the two subunits are associated, PE2 appears to protect E1 itself from reduction, possibly by promoting the export of the complex from the endoplasmic reticulum (Carleton *et al.*, 1997). It may be significant that expression of a temperature-sensitive E1 mutant at the restrictive temperature results in the retention in the endoplasmic reticulum of E1 and PE2 in disulfide-stabilized aggregates (Carleton and Brown, 1997). Most interestingly, the acquisition by E1 ϵ of its final metastable character appears to correlate with the processing of PE2 to E2 (Mulvey and Brown, 1994).

This entire line of investigation has been questioned (Glomb-Reinmund and Kielian, 1998). The weak base ammonium chloride and inhibitors of the vacuolar proton ATPase, bafilomycin A1 and concanamycin, prevent endosomal acidification. Incubation of cells with any of these reagents during the process of entry of either Semliki Forest virus or Sindbis virus inhibited infection. No inhibition was detected if the cells were

incubated with the reagents after entry. It was concluded that entry of both viruses requires uptake into endosomes and exposure to low pH. The authors were able to confirm the earlier finding that treatment of the virus and cells with 5,5'-dithiobis-(2-nitrobenzoic) acid during entry inhibited both Sindbis and Semliki Forest virus infection. However, incubation with 1 mM monobromotrimethylammoniumbimane did not reduce infection. In addition, treatment with 0.1 mM *p*-chloromercuriphenylsulfonic acid had only modest inhibitory effects on infection by both viruses. These results were contrasted with the more dramatic effects these reagents have on the reduction of HIV-1 infection (Ryser *et al.*, 1994) or diphtheria toxin intoxication (Ryser *et al.*, 1991). The authors concluded that entry occurs through the endocytic pathway and that blocking cell-surface sulphydryls did not substantially or specifically inhibit infection. They asserted that they had no evidence for a specific role for thiol-disulfide exchange reactions in Semliki Forest or Sindbis virus infection.

These experiments rule out the participation in alphaviral entry of the same cellular enzymes that play roles in HIV-1 infection and diphtheria toxin infection. The experiments do not, however, exclude the possibility of viral protein-mediated thiol-disulfide-exchange reactions. A model for alphaviral entry that incorporates both acidified endosomes and thiol-disulfide exchange will be presented following a discussion of the system in which there is the most detailed and well-supported proposed mechanism for the role of thiol-disulfide exchange in viral-glycoprotein-mediated membrane fusion.

3.4. Murine Leukemia Viruses

Examination of the available data on murine leukemia virus (MuLV) envelope proteins (Envs) indicates that disulfide-bond rearrangement plays a critical role in the induction by receptors of Env-mediated membrane fusion. The MuLV Envs are synthesized as single polypeptide precursors with cleaved signal sequences (Henderson *et al.*, 1984; Shinnick *et al.*, 1981). During its progress through the secretory system the Env protein forms a trimer (Kamps *et al.*, 1991) and is subsequently proteolytically processed into two subunits (Henderson *et al.*, 1984; Witte *et al.*, 1977), SU and TM, which are linked through a labile disulfide bond (Pinter *et al.*, 1978). SU is on the outside of the retrovirus particle, whereas TM possesses an extraparticle domain, a membrane-spanning domain, and a 35-amino-acid domain that resides within the particle (Pinter and Honnen, 1983).

The sequence of the MuLV Envs predicts that each SU protein will possess an even number of cysteine residues, whereas the extracellular

domain of TM will include just three, which are present in a sequence CX₆CC that lies at the end of a putative coiled-coil forming region. The cysteine residues are absolutely conserved among members of an MuLV group and many are conserved across group boundaries. Extracellular cysteines are normally found in disulfide bonds, so it is intriguing that there is an odd number of highly conserved cysteines in the Env extracellular domain. The TM cysteine residues and a CXXC motif present at the amino-terminal portion of the carboxy-terminal domain of the SU protein are conserved in sequence and placement even among the Envs of more distantly related retroviruses such as human T-cell leukemia virus type 1 (HTLV-1), bovine leukemia virus (BLV), and Mason-Pfizer monkey virus (M-PMV) (Figure 1A).

CXXC motifs are relatively rare, and are conserved virtually exclusively in two other classes of polypeptides, transition-metal binding proteins (Holden *et al.*, 1994; Sahlman and Skarfstad, 1983) and thiol-disulfide-exchange enzymes, such as DsbA, DsbB, thioredoxin and protein disulfide isomerase (PDI), where the two-cysteine motifs are the active sites of the enzymes (Freedman *et al.*, 1994; Holmgren, 1985). In the handful of other proteins where a CXXC motif is conserved at least one of the cysteines is modified (by palmitoylation, in the case of H-ras (Hancock *et al.*, 1989)) or the cysteines are each disulfide-linked to other cysteines in the protein (ovomucoid (Weber *et al.*, 1981) and surfactant protein B (Johansson *et al.*, 1991)).

The SU subunit CXXC motif is thus reminiscent of the active site of thiol-disulfide-exchange enzymes (Figure 1A), which function by the following mechanism: When the reduced exchange protein encounters a substrate disulfide, a mixed disulfide is formed between the first cysteine of the enzyme active-site CXXC and one sulfur atom of the substrate; the second substrate sulfur atom is reduced to a sulfhydryl. Next an intramolecular cystine bridge forms between the first and second cysteine of the active site of the enzyme, resulting in the reduction of the substrate disulfide to two sulfhydryls (Holmgren, 1985).

Analysis of the roles of the envelope-protein sequences in the context of the mechanism of the thiol-disulfide-exchange enzymes suggests an attractive model. In this model, the first cysteine in the SU subunit CXXC motif forms a disulfide with the last of the cysteines in the TM subunit CX₆CC motif, whereas the two other TM cysteines form an intramolecular cystine bridge (Fass *et al.*, 1996; Fass and Kim, 1995). The intermolecular disulfide rearranges to an intramolecular bond, *i.e.*, between the two cysteines of the SU subunit CXXC motif, upon receptor binding. Extrusion of the TM fusion peptide occurs via an irreversible spring-loaded mechanism

analogous to that proposed for the influenza virus hemagglutinin protein, and membrane fusion ensues (Figure 1B).

There are three lines of evidence suggesting that such a scenario may be correct. The first is that the SU-TM disulfide is labile in the presence of detergents, but it is stabilized by *N*-ethylmaleimide treatment prior to solubilization (Gliniak *et al.*, 1991; Pinter and Fleissner, 1977; Pinter *et al.*, 1997; Pinter *et al.*, 1978) or by reducing the pH during solubilization (Opstelten *et al.*, 1998). This suggests that the blocking of a free sulphydryl, presumably the second cysteine of the CXXC motif, stabilizes the preexistent intermolecular cystine bridge (Figure 1C). The second is that the products of protease digestion of the disulfide-linked Friend MuLV SU and TM indicates that the bond is between a half-cystine in the disulfide exchange motif and one in the CX₆CC motif (Pinter *et al.*, 1997). The third is that investigations of the disulfide bonds in the isolated Friend MuLV and Friend Mink-Cell Focus-inducing virus SU proteins indicate that the two cysteines in the CXXC motif are linked in a cystine bridge (Linder *et al.*, 1992; Linder *et al.*, 1994). Such cystine bridges are known to be highly strained and are found heretofore only in proteins with thiol-disulfide exchange activity (Darby and Creighton, 1995; Zapun *et al.*, 1993). The idea that thiol-disulfide exchange accompanies receptor binding is as yet unsupported by direct experimental data. However, it is plausible considering the conservation and placement of the motifs and the analogies with ricin and diphtheria toxin: Interchain disulfide bond reduction appears to be a rate-limiting step in membrane penetration by both toxins (Lewis and Youle, 1986; Papini *et al.*, 1993). In contrast to the HIV glycoproteins, which appear to be dependent on exogenous enzymatic activities for the catalysis of disulfide-bond rearrangements (Ryser *et al.*, 1994), the MuLV and other homologous Envs would possess a thiol-disulfide-exchange enzyme active site as part of their structures. Other than the regions of the two cysteine-containing motifs and a GXDP motif (Gallaher *et al.*, 1995), the MuLV envelope proteins share essentially no sequence identity with those of the more distant HTLV-1, BLV, and M-PMV, suggesting that these conserved motifs have coevolved and are likely to play similar roles in all cases. Although a covalent interaction between SU and TM has not been found for some of the more distantly related viruses (Brody *et al.*, 1994), these studies have not utilized conditions capable of stabilizing a labile disulfide bond.

It has been suggested that the SU-TM disulfide bond is not "labile" to reduction but merely appears to rearrange as an artifact of solubilization (Opstelten *et al.*, 1998). It is therefore, in fact, a "stable" disulfide when present in intact glycoprotein complexes on the surface of cells or virions. Nevertheless, the SU-TM linkage is "labile" in the sense that extra care is

A

Mo-MuLV

M-PMV

HTLV-1

BLV

Thioredoxin

Protein Disulfide Isomerase

Glutaredoxin

Thioredoxin Reductase

CWLC

CWLC

CIVC

CAIC

CGPC

CGHC

CPYC

CATC

GGLCAALKEECCF

GGICLALQEKCCF

GGLCKALQEQCCF

QSLCPTINEPCCF

CXXC

S—S

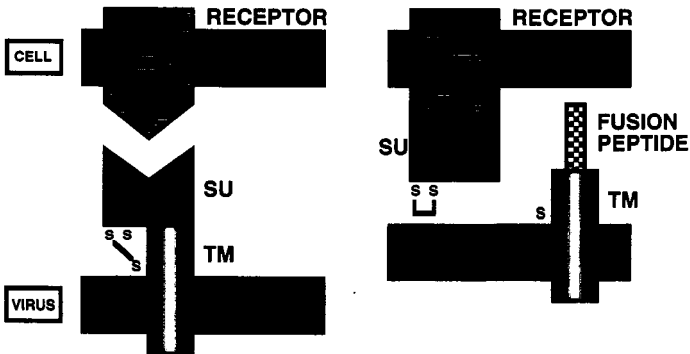
CXXC

S SH

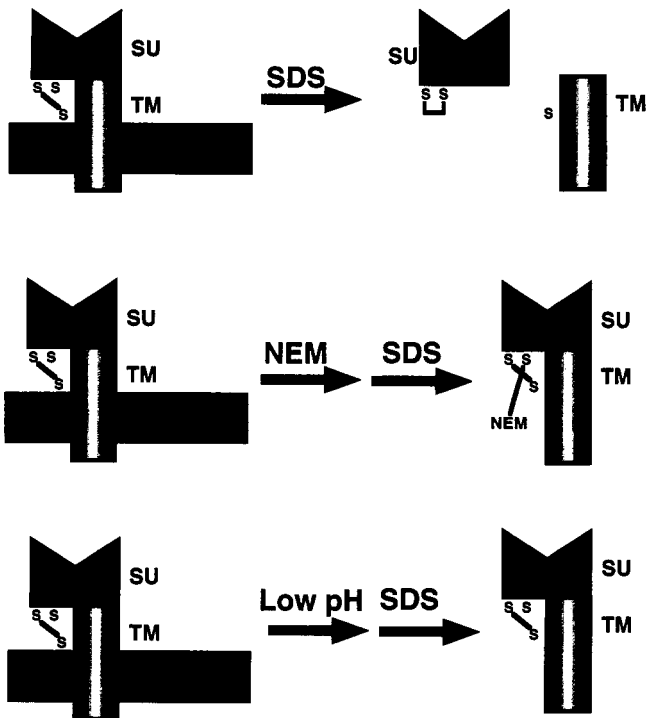
CXXC

SH SH

B



C



required to maintain it during analysis of the proteins (Figure IC) and that severance of the linkage may ensue during the conformational changes associated with membrane fusion.

The proposed thiol-disulfide-exchange mechanism suggests that there is a conformational coupling between receptor binding and the relative stability of the intermolecular versus intramolecular cystine bridge. It is possible that there is an equilibrium between the two disulfides and that when the SU protein binds to the receptor, the mobility of the receptor in the



FIGURE 1. Envelope-protein thiol-disulfide exchange motifs and receptor induction of membrane fusion.

(A) Comparison of the retroviral envelope protein sequences and those of the thiol-disulfide exchange enzymes and the catalytic mechanism of the enzymes. The conserved thiol-disulfide exchange motifs in the Envs of Moloney MuLV (AA 336–339), Mason-Pfizer monkey virus (AA 247–250), human T-cell leukemia virus type I isolate MT-2 (AA 225–228), and bovine leukemia virus (AA 212–215) and in the active sites of *E. coli* thioredoxin, rat protein disulfide isomerase, *E. coli* glutaredoxin (Fahey and Sundquist, 1991), and *E. coli* thioredoxin reductase (Russel and Model, 1988) are compared. Also shown are the three-cysteine motifs in the envelope TM subunits—Mo-MuLV AA 552–564 (Shinnick *et al.*, 1981), M-PMV AA 472–484 (Sonigo *et al.*, 1986), HTLV-1 MT-2 isolate AA 390–402 (Gray *et al.*, 1990), and bovine leukemia virus AA 380–392 (Sagata *et al.*, 1985).

The catalytic mechanism of thioredoxin (Holmgren, 1985) is depicted below. When the reduced thioredoxin (represented by its active site motif) encounters a disulfide, a mixed disulfide is formed between the first cysteine of the enzyme active site and one of the sulfurs of the substrate. An intramolecular cystine bridge then forms between the first and second cysteine of the active site of the enzyme, resulting in the reduction of the substrate disulfide to two sulfhydryls. It is the last two steps (ignoring the sulfhydryl in outline) that are similar to the proposed disulfide shuffling in Env.

(B) Model of thiol-disulfide exchange and receptor induction of membrane fusion. The first cysteine in the SU (black) CXXC motif initially forms a disulfide with one of the cysteines in the TM (shaded figure in viral membrane) CX₆CC motif. In this conformation the binding of SU to TM prevents exposure of the fusion peptide. There is a free thiol in SU.

The intermolecular disulfide rearranges to an intramolecular bond upon receptor (striped figure in cell membrane) binding. Extrusion of the TM fusion peptide (the checked rectangle at the end of the TM protein) and membrane fusion follow. Complete dissociation of the SU and TM subunits may not be necessary for membrane fusion; the model requires only that substantial subunit reorganization results from intermolecular cystine-bond cleavage.

(C) Fate of the Mo-MuLV Env intermolecular disulfide bond under different experimental conditions. When the Moloney murine leukemia virus is disrupted in SDS the disulfide bond between the two MuLV Env subunits rearranges in an experimental artifact during the process of denaturation. If the virus is pretreated with *N*-ethylmaleimide (NEM) before disruption (Pinter and Fleissner, 1977; Pinter *et al.*, 1997; Pinter *et al.*, 1978) or if the pH is lowered to pH 6.0 (Opstelten *et al.*, 1998), then the disulfide bond linking SU and TM is preserved. In the former case rearrangement is prevented by alkylation of the free cysteine (Pinter *et al.*, 1997), whereas in the latter case it is inhibited by reducing the concentration of the thiolate, which is the nucleophile that participates in the exchange reaction (Opstelten *et al.*, 1998).

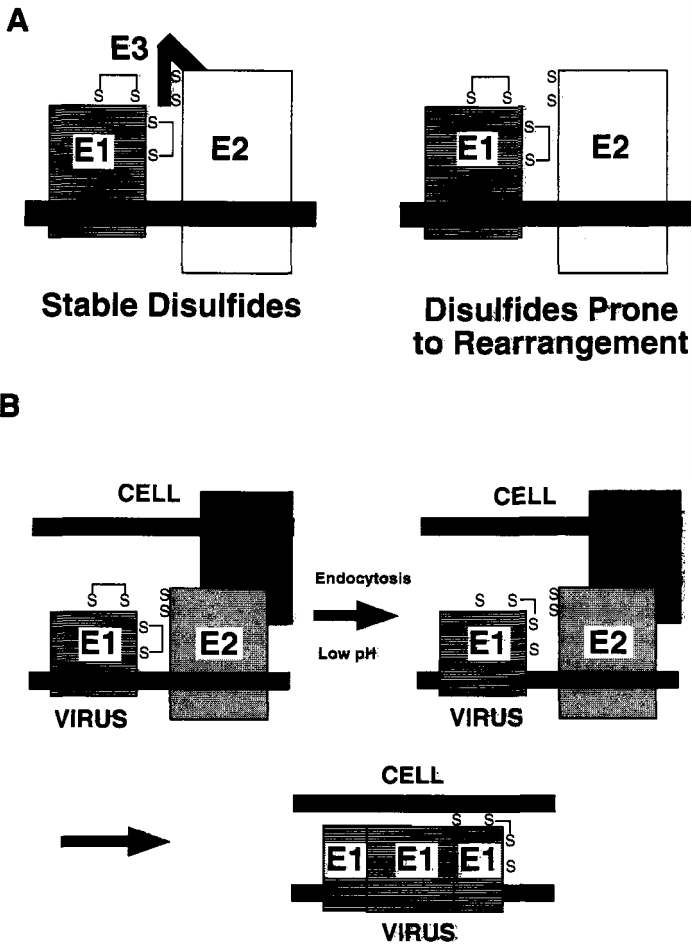


FIGURE 2. A model for alphavirus entry into cells.

(A) Alphaviral PE2 processing and activation of thiol-disulfide exchange. During the progress of the alphaviral glycoproteins PE2 and E1 (striped rectangle) associate to form a trimer of heterodimers. Only one heterodimer is illustrated for the sake of simplicity. PE2 consists of E3 (black) covalently linked to E2 (light-gray rectangle). The predicted E3 alpha helices (David A. Sanders, unpublished observations) overlie the E2 amino-terminal thiol-disulfide (CXXC) motif inhibiting its activity. The glycoproteins are transported to the Golgi apparatus where PE2 is cleaved into E3 and E2 at a sequence recognized by the furin class of protein convertases (Straws and Strauss, 1994). The cleavage is postulated to expose the thiol-disulfide exchange motif. This contributes to the instability of E1 with respect to its disulfide bonds during denaturation (Mulvey and Brown, 1994). There is no intention of implying that evidence for any particular configuration of oxidized or reduced cysteine residues exists within E1 and E2; one possibility is illustrated.

cellular membrane results in the stripping of the SU protein from the virus envelope-protein complex during the otherwise brief periods when the intramolecular disulfide is present. The irreversibility of the proposed TM conformational change leads to exposure of the fusion peptide and membrane fusion. It appears more likely that binding of the cellular receptor directly affects the conformation of protein segments that follow the receptor-binding domain sequence in the SU amino-terminal domain and that these in turn stabilize the intramolecular form of the disulfide relative to the intermolecular form.

3.5. Other Retroviruses and Filoviruses

Some retroviruses, such as the avian sarcoma and leukosis viruses (ASLVs), and the filoviruses contain a CX₆CC motif in their TM subunits in an equivalent position (at the end of a predicted coiled-coil forming region) to that of the MuLVs. However, they do not contain the CXXC motif in their SU subunits and have a stable disulfide linkage between their subunits rather than a labile one. It is noteworthy that both the ASLVs and the filoviruses are predicted to have internal fusion peptides within a cystine-bridged loop of TM rather than at the amino-terminus of TM, as is the case for the MuLVs (Gallagher, 1996). It is probable that the first two cysteine residues in the ASLV and filovirus TM CX₆CC motifs form an intramolecular disulfide bond while the third cysteine residue participates in an intermolecular cystine bridge with a residue from the SU subunits. In the case of the filoviruses, it appears possible that, the acidic environment of the endosome induces a conformational change in the GP during viral entry, which makes the filovirus SU-TM disulfide linkage more readily reduced by cellular factors, which in turn leads to dissociation of the subunits and membrane fusion. The filoviruses, and potentially other viruses, would thus be dependent during entry upon cellular factors for elimination



(B) Activation of thiol-disulfide exchange on alphaviral glycoproteins in endosomes. An alphavirus binds to a cellular receptor (dark-gray polygon) through E2. Conformational changes in the glycoprotein complex transpire and the virus is taken into the cell by endocytosis. The acidity in the endosomes produce structural changes in the glycoproteins that alter the accessibility of E1 disulfides to the E2 thiol-disulfide exchange motif. This leads to reduction of particular cystine bridges and/or formation of novel disulfide linkages. Again, there is no intention of implying that evidence for any particular configuration of oxidized or reduced cysteine residues exists within E1 and E2. The disulfide rearrangements accompany or lead to the fusion-promoting conformation of E1, which probably involves dissociation of E2 from E1 and formation of an E1 homotrimer (Wahlberg *et al.*, 1992). The pK_a of one or both of the cysteines in the CXXC motif could be altered so that thiol-disulfide exchange is promoted even in acidic endosomes (Wells *et al.*, 1993).

of the covalent bond, whereas the retroviruses whose Envs are most closely related to those of the MuLVs would encode their own enzymatic activity. One possible alternative scenario for the filovirus GPs is that reduction of the disulfide bond is not required for membrane fusion but does enhance its efficiency. It has been suggested that acid-induced conformational changes may facilitate the reduction of the cystine bridge between the two subunits in cleaved influenza hemagglutinins (Roberts *et al.*, 1993).

3.6. A Reconsideration of Alphavirus Entry

Thiol-disulfide exchange may be a common biochemical step in the conformational changes that viral glycoproteins undergo during membrane fusion. Reexamining the mechanism of entry of alphaviruses in this context indicates that a hypothesis that includes a role for thiol-disulfide exchange is plausible. Three sets of facts favor this proposition. First, rubella virus, which is related to the alphaviruses (together with which it forms the togavirus family) possesses an E1 glycoprotein that contains a CXXC motif. Intriguingly, the two cysteines in the motif are linked in a cystine bridge (Gros *et al.*, 1997). As pointed out above, such disulfide bonds are found only in proteins with thiol-disulfide exchange activity. Second, the E2 glycoprotein of alphaviruses also possess a conserved CXXC motif. It is positioned at the amino-terminus of E2 approximately 20 amino-acid residues after the E3/E2 cleavage site. Third, the previously noted effects of 2-mercaptoethanol on Sindbis virus-mediated membrane fusion and of 5,5'-dithiobis-(2-nitrobenzoic) acid on Sindbis virus entry, and the acquisition of disulfide-bond metastability by E1 at the time that E3 is cleaved from E2 suggest that thiol-disulfide exchange is having an effect on glycoprotein conformation and function.

Synthesizing these data leads to the following model (Figure 2). When an alphaviral glycoprotein is progressing through the secretory system as a PE2-E1 complex, the activity of the amino-terminal PE2 CXXC peptide region is inhibited by the presence of E3. Upon cleavage of E3 the CXXC region is exposed so that it is poised to engage in the promotion of thiol-disulfide exchange during viral entry (Figure 2A). Binding of the alphavirus to its cellular receptor(s), presumably through E2, leads to some conformational changes in the glycoproteins. The virus is taken into the cell by clathrin-mediated endocytosis. Upon exposure of the receptor-bound glycoproteins to low pH, conformational changes occur that expose one or more disulfide bonds, perhaps in E1, to the E2 thiol-disulfide exchange (CXXC) motif. Rearrangement of the disulfide bond(s) leads to the structural changes that result in a fusion-promoting conformation of E1, which probably involves dissociation of E2 from E1 and formation of an E1

homotrimer (Figure 2B) (Wahlberg *et al.*, 1992). It is noteworthy that cleavage of PE2 appears to be necessary for the dissociation of the heterodimer under normal conditions (Salminen *et al.*, 1992). Perhaps E1 and PE2 form a difficult-to-detect cystine bridge, similar to the MuLV SU-TM disulfide bond, that cannot rearrange under normal conditions, but does rearrange during solubilization of the proteins.

4. CONCLUSION

Although much of the evidence for the roles of protein thiols and thiol-disulfide exchange in membrane fusion is indirect, it appears probable that additional cellular membrane fusion-promoting proteins containing catalytically important or thiol-reagent-accessible cysteine residues, besides the *N*-ethylmaleimide-sensitive factors and cysteine proteases, will be identified. Of equal importance and probability will be the ongoing investigation of the occurrence and prevalence of thiol-disulfide exchange reactions in viral glycoproteins during the process of membrane fusion. Such studies may not only illuminate a critical and common biochemical step in membrane-fusion but may also lead to the identification of reagents that can specifically inhibit the thiol-disulfide exchange and thereby prevent viral entry.

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